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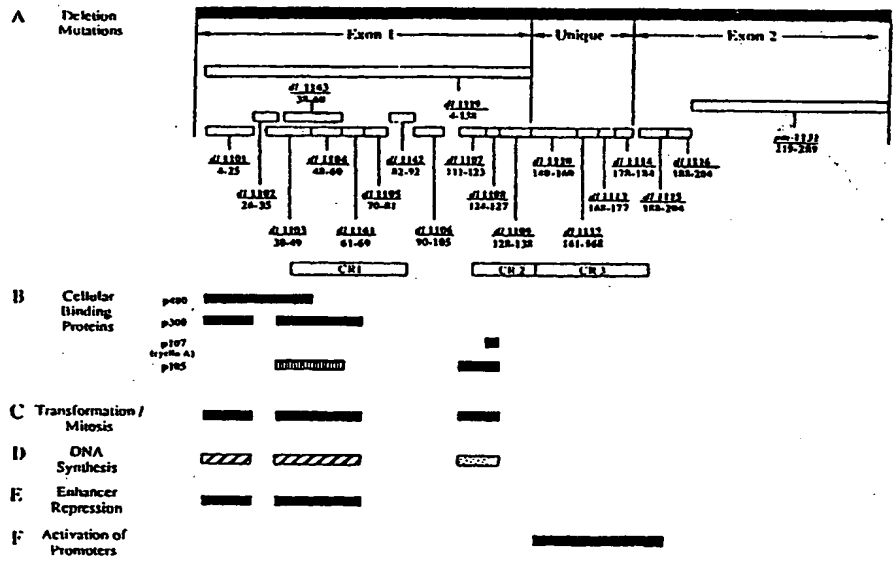

(57) Abstract

The present invention is directed to recombinant adenoviral vectors capable of replication under particular host cell conditions. In particular, the present invention provides adenoviruses containing modifications to the E1a region which have therapeutic and diagnostic applications. The vectors of the present invention are capable of replication and lysis of neoplastic cells. The vectors may optionally include modifications to the genome so as to impart specific replicative or targeting functions. The present invention also provides pharmaceutical formulations of such vectors. The present invention further provides methods of use of such vectors. The present invention further provides methods of preparing the vectors.

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TITLE

RECOMBINANT E1A DELETED ADENOVIRAL VECTORS

BACKGROUND OF THE INVENTION

The adenovirus E1 region, which encodes the immediate early gene E1a and the early gene E1b, plays a key role in the adenovirus life cycle and is responsible for interfering with the ability of the infected cell to regulate cell cycle progression, differentiation and programmed cell death (apoptosis). The E1a gene products stimulate infected cells, which are normally differentiated and quiescent, to progress into the S-phase of the cell cycle in order that viral DNA replication can occur. Normal cells usually respond to unscheduled stimulation of cell cycle progression (by E1a or other mitogenic factors) by activation of p53-dependent apoptosis. However, in the context of a viral infection the E1a products do not stimulate apoptosis because the protein products of the E1 region gene, E1b, are effective inhibitors of apoptosis. Therefore, during the early stage of viral infection the E1a and E1b gene products cooperate to bring about a quasi tumorigenic state in the infected cell which is required for efficient viral DNA replication and a productive infection cycle. The full scope of activity of the adenovirus E1a region is described in Bayley, S. and Mymryk, J. (1994) Intl. J. of Oncology 5:425-444. For a comprehensive review of the adenovirus biology see Shenk, T. (1996), *Fields Virology*, 3rd Edition: p2111-2148.

The E1 genomic sequence is located at the extreme right end of the 36kb adenoviral genome. The primary E1a mRNA is differentially spliced during the early phase of replication into two prominent mRNAs, called 13S and 12S, which give rise to 289R and 243 AA proteins, respectively (see Figure 9 of the attached drawings). The 289R and 243R proteins differ only by an internal sequence of 46 amino acids that is unique to the larger protein. Although the E1a primary transcript is spliced into to 3 other mRNAs called 11S, 10S, and 9S, which encode for proteins of 217R, 171R and 55R respectively, these messages and not made efficiently in the early phase of infection and it is likely that the 289R and 243R proteins carry out the primary functions

of E1a during the viral life cycle. The E1b gene transcript is also differentially spliced to yield mRNAs of 22S, 14.5s, 14S, and 13S each of which contains two open reading frames. One of these open-reading frames is common to all of the messages and encodes a protein of 179R. Depending on the mRNA, the other open reading frames give rise to proteins of 84R, 93R, 155R and 496R. Of the E1b proteins the 176R and 496R proteins, which are also referred to as E1b 19K and E1b 55K respectively, are the most prominent and best characterized.

The E1a and E1b gene products play critical roles in the productive infection cycle to prepare the infected cell for viral replication and to regulate viral specific processes. The E1a and E1b products do not contain intrinsic enzymatic activities, but are thought to carry out their functions by interacting with a number of cellular proteins. The E1a proteins associate with a wide range of cellular proteins including p400, p300, cAMP-responsive transcription binding protein (CBP), p130, p107, pRb, cyclin A, cdk2 and TATA-binding protein (TBP). Mapping studies have been used to compare cellular protein binding domains and functional domains in the E1a proteins (see Figure 10). Stimulation of cell cycle progression by E1a has been mapped to three regions in the common amino terminal domain of the 243R and 289R proteins (Howe *et al.*, (1990) PNAS 87:5883-5887). These regions are commonly referred to as the amino-terminal domain, conserved region 1 (CR1) and conserved region 2 (CR2). The amino-terminal domain and CR1 are required to bind a number of proteins including p300/CBP which are thought to be co-activators of gene transcription that have been implicated in regulation of cell proliferation and differentiation. The third E1A region required for cell cycle regulation by E1a is CR2 which is required for association with the know members of the pRb family of related cell cycle regulators including pRb, p107 and p130.

The pRb family members regulate the cell cycle by binding to a the E2F class of transcription factors that in turn regulate expression of genes that are required for cell cycle phase transitions. Binding of p300 and the pRb family members appears to inactivate the ability of these proteins to suppress cell cycle progression and this appears to be the major mechanism by which E1a induces resting cells to progress into the cell cycle. A large body of evidence has accumulated to support this hypothesis. For

example, by associating with pRb the E1a proteins disrupt E2F-pRb complexes which frees E2F to stimulate gene expression that allows progression into the S-phase of the cell cycle. It is not precisely known how p300 regulates cell proliferation, but p300 is known to regulate expression of genes that are required to maintain a differentiated phenotype and that inhibition of p300 can block terminal differentiation. In addition, it is known that E1a-mutants that associate with p300, but are defective for binding pRb, are nevertheless able to stimulate phosphorylation of pRb which leads to disruption of pRb-E2F complexes and cell cycle progression (Wang *et al.*, (1991) Mol. Cell. Bio. 11, 4253-4265).

Induction of unscheduled DNA synthesis by E1a is a cellular stress that is sensed by the infected host cell. The infected cell responds by inducing apoptosis which is normally mediated by p53. P53 is activated in response to a wide variety of cellular stresses including DNA damage, hypoxia and expression of mitogenic oncogenes including E1a. Productive viral infection cannot occur if the infected cell commits programmed cell death and therefore the virus has evolved to inhibit apoptosis, at least early during the infectious cycle, by production of the E1b 19K and 55K products(for a review of regulation of apoptosis by E1b see White, E., 1998, Seminars in Virology 8, 505-513). The E1b 19K is considered to be the primary inhibitor of E1a-induced apoptosis because E1b 19K alone blocks E1a induced apoptosis more efficiently than E1b 55K alone. The E1b 19K inhibits apoptosis by two different mechanisms. First, E1B 19K associates with the proapoptotic Bcl-2 family members Bax, Nbk/Bik and BNIP3, and inhibit the ability of these proteins to induce apoptosis. Second, the E1B 19K protein can inhibit apoptosis by interacting with factors such as FADD and CED4 which normally act to activate caspases for apoptosis. The E1b 55K protein binds to inhibits the ability of p53 to act as an activator of transcription and can therefore augment the ability of the E1B 19K protein to inactivate p53-dependent apoptosis.

In addition to stimulating cell cycle progression and suppressing apoptotic pathways the E1a and E1b proteins also play important viral specific roles during the replication cycle of the virus. The E1a protein s initiate the coordinated expression of the viral genome by stimulating expression of promoters for the E1b gene in addition to

the other early gene regions E2, E3 and E4. The 289R E1a protein is primarily responsible for the transactivation of the early adenoviral promoters and mapping studies have shown that the 46 amino acid unique domain of the 289R protein plays the major role in activation of the early viral promoters. The E1b 55K protein also carries out critical viral specific roles during the productive infection cycle. Adenovirus E1b 55K mutants are defective for late viral protein production and shutoff of host cell protein synthesis, consequently these mutant viruses are defective for growth on a number of human cell lines (Babiss and Ginsberg, (1984) J.Virol. 50:202-212; Babiss *et al.*, 1985 Mol. Cell. Biol. 52552-2558; Pidler *et al.*,(1986) Mol.Cell.Biol.6:470-476; Yew *et al.*,(1990) Virology 179:795-805). More recently it has been suggested that the E1b55K may alter cell cycle controls in infected cells (Goodrum and Ornelles, 1997 J. Virol. 71, 548-561) and in addition E1b55K may influence viral DNA replication (Ridgway *et al*, (1997) Virology 237:404-413).

Attempts have been made to exploit the ability of the E1b 55K protein to bind p53 in the design of adenoviruses that selectively replicate in and kill p53 deficient cells by the elimination of E1b 55K function. See McCormick, United States Patent No. 5,677,178 issued October 14, 1997. The vectors have been under commercial development by ONYX Pharmaceuticals. A particular vector, ONYX-O15 contains a deletion in the p55 coding sequence. This prevents the expression of a E1b55K product capable of binding p53 and is claimed to result in preferential replication of the virus in p53 deficient tumor cells. However, a number of reports, in addition to data presented below, have brought the replication specificity of the E1b 55K viruses for p53 deficient tumor cells into question. Goodrum and Ornelles (1997) J. Virol. 71, 548-561 have suggested that the E1b 55K proteins relieve growth restrictions imposed on viral replication by the cell cycle and that the ability of E1b55K mutant viruses to replicate is not mediated by the status of p53. In addition, other studies have suggested that the interaction between p53 and E1b 55K may be required for efficient viral replication (Ridgway *et al.* (1997) Virology 237:404-413). Data presented below extend these observation by demonstrating that E1b55K mutant viruses are defective for viral growth in normal cells, but also in a variety of tumor cell lines regardless of the status of the p53 gene. Together these observations suggest that E1b 55K mutant viruses are

growth defective in all cell types and do not target p53 defective tumor cells for selective cell killing. Therefore there is a need for replication competent adenovirus vectors that target tumor cells specifically.

Alternative to the idea of selectively replicating vector is the employment of a replication deficient adenoviral vector containing extensive elimination of E1 function. In particular, vectors containing elimination of E1, E2, E3 and partial E4 deletions have been employed to delivery exogenous transgenes. Such vectors have been employed to deliver the p53 gene to target cells. It has been demonstrated that the expression of an exogenously administered wild type p53 in a p53 deficient (p53 mutated or p53 null) tumor cell is capable of inducing p53 mediated apoptosis in the tumor cell. Such viral vectors for the delivery of p53 are currently under development Schering Corporation and Introgen Corporation. Again these vectors have demonstrated acceptable toxicology profiles and therapeutic efficacy for human therapeutic applications and are in Phase II clinical trials in man for the treatment of p53 related malignancies.

Replication deficient and selectively replicating vectors have, at least in theory, design drawbacks which are of concern to clinicians. Because the replication deficient vectors will not propagate uncontrollably in the patient, they have a more theoretically appealing safety profile. However, as effective tumor elimination requires the infection of the substantial majority of the tumor cells being infected, a substantial molar excess of vector is commonly used to insure therapeutic effectiveness. Selectively replicating vectors are viewed as being more of an issue from a safety perspective because of their ability to replicate and potentially mutate to form fully replication competent vectors in the patient. However, exploiting the natural ability to the virus to propagate under particular conditions enables these vectors to spread to surrounding tumor cells. Since the vectors themselves are able to replicate, a lower initial dose of such vectors is required. This is favorable from an immunological perspective as well as for economic reasons in the manufacture of such agents.

Therefore, there is a need in the art for a selectively replicating vector that addresses the perceived safety problems while providing the increased therapeutic index. The present invention solves these problems by providing a selectively replicating adenovirus vector containing particular E1a modifications such that the

vector replicates preferentially in rapidly growing non-differentiated or dedifferentiated cells. The present invention also provides pharmaceutical formulations comprising such vectors. The present invention also provides methods of eliminating tumor cells from a population of normal cells by using such vectors.

SUMMARY OF THE INVENTION

The present invention provides a recombinant adenoviral vector containing modifications to the E1a coding sequence so as to eliminate the ability of the E1a gene product to bind to the p300/CBP and/or related proteins, and the Rb protein family, while retaining the transactivating function of the E1A CR3 domain. The E1A proteins associate with p300/CBP, in addition to pRb, to down regulate differentiation and cell growth control pathways in the normally quiescent differentiated cells that are infected by wild type adenovirus. Binding of these cellular regulatory proteins by E1A results in a quasi-tumorigenic state in the infected cell that is required for productive adenoviral infection. This invention is intended to target rapidly dividing dedifferentiated cells (such as tumor cells), as opposed to growth arrested and/or differentiated cells, by attenuating E1A functions that deregulate cellular regulatory path vays. These constructs will preferentially replicate in cell types, such as tumor cells, in which cellular growth and differentiation pathways are disrupted. The invention further provides pharmaceutical formulations and methods of use of same. The present invention also provides method of making such vectors and formulations.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a microscopic (100X) view of C33A cells were infected with the indicated viral constructs at a concentration 1.8×10^9 particles per ml and stained with crystal violet.

Figure 2 represents experimental results similar to those presented in Figure 1 except that the viral concentration was 1.8×10^8 particles per ml.

Figure 3 represents experimental results similar to those presented in Figure 1 except that the viral concentration was 1.8×10^7 particles per ml.

Figure 4 is a microscopic (100X) view of MRC9 cells were infected with the indicated viral constructs at a concentration 1.8×10^9 particles per ml and stained with crystal violet.

Figure 5 represents experimental results similar to those presented in Figure 4 except that the viral concentration was 1.8 x 10^8 particles per ml.

Figure 6 represents experimental results similar to those presented in Figure 4 except that the viral concentration was 1.8 x 10^8 particles per ml.

Figure 7 is a histogram which illustrates that the production of virus at 48 hours post infection in various cell lines as indicated. Cells were infected at a particle concentration 1.8 x 10^9 particles per ml.

Figure 8 represents the experimental results similar to those presented in Figure 7 except that the viral concentration was 1.8 x 10^8 particles per ml.

Figure 9 is a map illustrating the differential splicing of the E1A message.

Figure 10 is a graphical representation of the various mutations in the E1a coding sequence and the phenotypes associated with the respective mutants.

Figure 11 is a graphical presentation of data relating to the effective dose required to achieve cell killing in normal lung endothelial cells (Panel A) and the A549 lung endothelial tumor cells (Panel B). The vertical axes represent the percent of uninfected controls and the horizontal axes represent the viral dose in particles per milliliter. The experiments were performed by exposing a culture of each cells o six different concentrations of virus from 10^6 to 10^{10} viral particles. The viruses used in these experiments were the dl309 virus (filled squares), the dl01/07/309 virus (filled circles), the E1Bdl55K virus (open circles), and control virus (open squares) containing a deletion of the E1a and E1B regions and referred to as the A/C/N virus described in Wills, et al. (1994) Human Gene Therapy. The cells were exposed to the virus for a period of one hour, the excess virus washed and the percent of viable cells at six days following infection was determined the MTS assay (Promega, Madison WI) in substantial accordance with the manufacturer's instructions. The horizontal dotted line represents the level at which 50% of the cells remained viable. The intersection of the curves generated by the data and the horizontal dotted line is a measure of the ED_{50} of the virus.

Figure 12 is a microscopic (100X) view of MRC-9 transformed normal cells (Panel A) and DLD-1 tumor cells (Panel B) infected with viral constructs at varying concentration (1.8 x 10^6 to 1.8 x 10^9 for DLD-1 cells and 1.8 x 10^7 to 1.8 x 10^{10} for normal cells) of virus as indicated and stained with crystal violet. The first column in each panel is the dl309 virus. The second column in each panel is the dl01/07/309

virus. The third column in each panel is the dl1101 virus. The fourth column in each panel is the dl1107 virus. The fifth column in each panel is the E1bdl55K virus. The sixth column in each panel is the control virus (A/C/N). In Panel A the top row represents a dose of 1.8×10^{10} particles/ml, the second row represents a dose of 1.8×10^{9} particles/ml, the third row represents a dose of 1.8×10^{8} particles/ml, the bottom row represents a dose of 1.8×10^{7} particles/ml.). In Panel B the top row represents a dose of 1.8×10^{9} particles/ml, the second row represents a dose of 1.8×10^{8} particles/ml, the third row represents a dose of 1.8×10^{7} particles/ml, the bottom row represents a dose of 1.8×10^{6} particles/ml.

Figure 13 is a graphical representation of data obtained from a nude mouse tumor model study to evaluate the anti-tumor effects of intratumoral administration of viruses. On Day 1 of the study, nude mice were injected subcutaneously with $5x10^{6}$ DLD-1 tumor cells and tumors allowed to form for approximately 12 days until tumors reach a volume of approximately 200 mm^3. Mice were injected intratumorally with a dose of $2.5x10^{9}$ viral particles in a volume of 100 microliters of each of the viruses rAd-Con (filled triangles); E1Bdl55K (open squares), dl309 (open circles) and dl01/07/309 (filled squares) and vPBS (filled circles) as a buffer control on days 12,13,14,15, and 16. The tumor sizes were evaluated on days 17, 25 and 32 of the study. The vertical axis represents mean tumor volume in cubic millimeters and the horizontal axis represents the day of the study.

Figure 14 is a graphical representation of data obtained in a nude mouse model study to evaluate the anti-tumor effects of intravenous systemic administration of viruses. On Day 1 of the study, nude mice were injected subcutaneously with $5x10^{6}$ PC-3 (prostate carcinoma) tumor cells and tumors allowed to form for approximately seven days until tumors reached a volume of approximately 50-60 mm^3. Mice were injected intratumorally with a dose of 1×10^{10} viral particles in a volume of 200 microliters of each of the viruses E1Bdl55K (open circles), dl309 (filled squares) and dl01/07/309 (filled circles) and vPBS (open squares) as a buffer control on days 7, 8, 24, 25, and 16 of the study. The tumor sizes were evaluated on days 7, 21, 29, 35 and 42 of the study. The vertical axis represents mean tumor volume in cubic millimeters and the horizontal axis represents the day of the study.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of ablating neoplastic cells in a population of normal cells contaminated by said neoplastic cells by the administration to said population of cells a selectively replicating recombinant adenovirus which contains modifications to the E1a coding sequence so as to produce E1a gene products which are deficient in binding to one or more p300 protein family members and one or more Rb protein family member protein but expresses a modified 289R protein retaining the transactivating function of the E1a CR3 domain.

The term "neoplastic cell" is a cell displaying an aberrant growth phenotype characterized by independence of normal cellular growth controls. As neoplastic cells are not necessarily replicating at any given time point, the term neoplastic cells comprise cells which may be actively replicating or in a temporary non-replicative resting state (G1 or G0). Localized populations of neoplastic cells are referred to as neoplasms. Neoplasms may be malignant or benign. Malignant neoplasms are also referred to as cancers. The term cancer is used interchangeably herein with the term tumor. Neoplastic transformation refers the conversion of a normal cell into a neoplastic cell, often a tumor cell.

The term "selectively replicating" refers to a recombinant adenoviral vector capable of preferential replication in a cell in one phenotypic state relative to another phenotypic state. Examples of different phenotypic states would include the neoplastic phenotype versus a normal phenotype in a given cell type. A vector which is "selectively replicating" will replicate in and kill neoplastic cells at least 10 fold more efficiently than the same virus in a non-transformed normal cell of the same tissue cell type at a dosage level which is sufficient to induce substantial cell death in tumor cells but not normal cells. The effect of dosage is an important consideration when determining whether a given recombinant adenovirus results in preferential neoplastic cell killing because at a sufficiently high dose almost any adenovirus, regardless of the degree to which its genome has been modified, will be cytotoxic due simply to the effects of the presence of the viral proteins such as hexon which is known to be cytotoxic. Similarly, even though the scientific literature may refer to an E1 mutant adenovirus as "replication defective" (suggesting that the virus is absolutely incapable

of replication in the absence of a cell line capable of complementing the E1 defect), such viruses are more accurately described as "attenuated for replication" because even viruses containing a deletion of the entire E1 region will replicate to some degree, particularly in cycling or rapidly dividing cells. As Mulligan observed (1990, Science 260:926-932):

> Although the expression of the E1 region has been shown to affect the expression of other viral gene products necessary for replication (*citing* Horwitz, M. in Virology, B.N. Fields Ed. (Raven, New York, 1990) Chapter 60)), the required of E1 gene expression for viral replication does not appear to be absolute. The early characterization of E1-deficient viruses demonstrate that at high multiplicities of infection, the E1 region was dispensable for replication (citing Jones and Shenk (1979) PNAS(USA) 76(8):3665-3669).

Consequently, the effect of viral dose cannot be ignored when determining whether or not a virus is truly a selectively replicating virus. A virus containing extensive deletions of E1 may appear to possess selectivity because they will replicate under certain conditions and certain dosages. For example, the dl312 adenovirus (Jones and Shenk, 1979, PNAS(USA) 76(8):3665-3669) contains a deletion of nucleotides 448-1349 which results in elimination of all E1a functions. The sequences encoding the 289R protein begins at nucleotide 560 and ends at approximately nucleotide 1542. The nucleotide sequence encoding the entire 289R protein including the CR3 transactivation domain is completely absent in this construct. As Jones and Shenk observed when dl312 was used to infect HeLa cells (which are transformed cervical carcinoma cells), "no RNA species corresponding to early regions 2, 3 or 4 were detected" clearly indicating that the transactivation function of the E1a 289R protein which is responsible for transactivating the E2, E3 and E4 genes was absent. However, at a higher dose (multiplicity of infection), replication of this virus was seen. Additionally, it should be remembered that HeLa cells are cycling neoplastically transformed cells which, although genotypically positive for pRb105, possess the HPV 18 E6 and E7 functions which degrade pRb105 thus rendering HeLa cells phenotypically negative for p105Rb.

Furthermore, in order to determine if an E1 mutant adenovirus is truly selective, it is necessary to evaluate the ability of the virus to replicate in tumor cells as compared to normal cells of the same tissue cell type and not transformed or immortalized cell lines which are already cycling. This is clear based on our understanding of the function of E1a adenoviral proteins. The primary purpose of the

E1a proteins is to force the normally quiescent cell into the cell cycle. This first step following viral infection is essential in for viral replication in quiescent cells because adenoviruses require factor present only in S-phase to achieve efficient viral replication. However, if a cell has already entered the cell cycle (such as an immortalized or transformed cell line), the effect of the E1a deletions will be, to some degree, obscured. Thus, in order to truly assess neoplastic cell selectivity, it is necessary that the comparison be made with normal cells versus neoplastic cells. Additionally, wild-type vectors may also appear to replicate selectively in tumor cells relative to normal cells early after infection because the cell is already cycling, however this apparent selectivity diminishes over time once the virus has stimulated the cell cycle. Consequently, the time following infection when selectivity is measured must sufficient to avoid this initial replication lag in normal cells.

The commonly used parameter ED_{50} (which is defined as the dose sufficient to induce cell death in 50% of the cells) provides an appropriate basis of comparison. The ED_{50} of a virus can readily be determined by typical dose escalation experiments in vitro. In order to ensure the most consistent basis of comparison, the ED_{50} is most appropriately expressed relative to a viral control to minimize the effects of variations of infectivity between the normal and neoplastic cell types and any assay variations. Consequently the unitless ratio: ED_{50}(virus)/ED_{50}(control) is used to express the relative toxicity of the virus in the cell and will be referred to as the "relative toxicity index" or "RTI." For purposes of the present invention, the "selectivity index" of a given virus is expressed by the ratio: RTI(tumor cells)/RTI(normal cells). Selectively replicating vectors will have a selectivity index of at least 10 and preferably much greater. For example, the selectively replicating vector dl01/07 was evaluated for its ability to replicate in and kill A549 cells (a lung tumor cell line) and in normal lung endothelial cells with appropriate viral controls. The results of these experiments are presented in Figure 11 of the accompanying drawings. The following table summarizes the data presented:

Table 1. Summary of RTI and Selectivity Indices of Viruses			
Virus	**RTI (normal cells)**	**RTI (tumor cells)**	**Selectivity Index**
rAd-Con	1.0	1.0	1.0
dl309	450	900	2.0
dl01/07/309	1.8	225	125
E1Bdl55K	40	30	1.33

As can be seen from the data presented, the dl309 (wild-type) virus possesses essentially no selectivity. The slight increase in replication shown in tumor cells relative to normal cells is expected as the cycling tumor cells will facilitate viral replication. Furthermore, the E1Bdl55K virus possesses essentially no selectivity. However, the 01/07/309 virus possesses a high selectivity index (125) demonstrating it possesses high selectivity for replication in tumor cells relative to normal cells of the same histological type.

In order to achieve a selectively replicating adenovirus, it is essential that the ability of the 289R and 243R proteins to bind to pRb105 and p300 both be deleted while maintaining the functionality of the CR3 transactivation domain of 289R. The CR3 domain is present only in the E1a 289R polypeptide as the 243R protein does not possess the transactivational activity of the 289R protein. Retaining the transactivational activity of the 289R protein is essential for efficient viral replication. While retaining the CR3 domain is essential it is not sufficient on its own. For example the dl1010 virus described in Whyte, et al (1989) Cell 56:67-75, contains a deletion of amino acids 2-150 of the 289R protein replacing them with a single glycine residue. This deletion eliminates the p300 and pRB105 binding domains of the E1a 289R gene product and retain the CR3 domain (although the effect of the deletion of amino acid on CR3 function may be questioned by virtue of the deletion of the Val147 and Pro150 in view of the pm1120 and pm1122 point mutation viruses described in Jelsma, *et al.* (1988) Virology 163:494-502). However, the dl1010 virus will not retain the tranactivational functions of the CR3 domain because the deletions to the 289R protein

are too extensive. Jelsma, et al. confirm this by testing an E1a mutant (dl1119) containing a lesser deletion of amino acids 4-138 of the 289R protein (which will not disrupt the Val147 and Pro150 residues arguably within the CR3 domain) for its transactivational activity. The results presented in Table 2 of the reference and the comments of the authors indicate that this E1a molecule possesses "essentially no ability to transactivate." Consequently, large deletions in the E1a region upstream of the CR3 domain will result in a mutant 289R gene product which is so conformationally disrupted that even though the CR3 domain is expressed it is not functional.

Similarly, it is essential that the p300 and p105 Rb binding be eliminated as elimination of each on its own is insufficient to confer selectivity. For example, a CPE assay was performed to evaluate the ability of replication competent adenoviruses containing deletions in p300 binding (dl1101) and pRb (dl1107) were compared with the dl01/07 adenovirus (which deletes both functions) to kill DLD-1 tumor cells and a MRC-9 "normal" cell line. Appropriate control viruses rAd CON (or ZZCB containing a deletion of the entire E1a and E1b regions), dl309 (a phenotypically wild-type virus containing deletions in the non-essential E3 region) and E1B55K (a recombinant virus containing a deletion in the 55K binding region of the E1b55k) were included. The results are presented in Figure 12 of the attached drawings. As can be seen from the data presented, at an equivalent dose (particle concentration) the dl1101 and dl1107 viruses were capable of killing the normal (MRC9) cells essentially as efficiently as wild type virus. Similar results were obtained with respect to the tumor (DLD-1) cells. However, if one compares the performance of the dl01/07 virus in the normal cell line and tumor cell line, the dl01/07 virus killed tumor cells as efficiently as the wild-type virus but was toxic to the normal cells only at dose equivalent to the E1 defective control virus. Furthermore, the dl01/07 virus was substantially less toxic to the normal cells when compared to the dl1101 and dl1107 viruses. Consequently, selectivity is determined not just by the deletion of Rb 105 binding but also by the elimination of p300 binding.

The deletions in the E1a 289R coding sequence necessary to achieve elimination of p300 and pRb binding are preferably as minimal as possible to prevent major disruption of the secondary and tertiary structure of the E1a 289R protein. In order to eliminate p300 binding it is preferred that a mutation be introduced in the DNA sequence encoding the p300 binding domains of 289R. Deletions of less than about 30 amino acids in the C-terminal region to eliminate p300 binding are preferred, although

smaller modifications are preferred. The deletion of amino acids 4-25 of the 289R protein are sufficient to disrupt p300 binding without affecting transactivational functions of CR3. For example, a deletion of amino acids from about amino acid 30 to amino acid 49 (dl1103) and more particularly 36 to 49 are alternatively preferred to eliminate p300 binding. Point mutations sufficient to disrupt binding p300 are particularly preferred. For example, a point mutation of the second amino acid from arginine to glycine (Arg2 → Gly2) in the 289R protein has been demonstrated to disrupt p300 binding (See e.g., pm563, Whyte, et al, (1989) Cell 56:67-75). Similarly, in regard to eliminating pRb105 binding, minimal modifications are preferred. Elimination of selective amino acids in the pRb105 binding domain such as amino acid 111-123 (dl1107) and amino acids 124-127 (dl1108) are preferred. Deletion of amino acids 111-123 (dl1107) is particularly preferred in that it retains the p107 binding activity of the 289R protein.

The dl01/07/309 virus is a particularly preferred embodiment of the present invention because although it deletes the p300 and pRb105 binding regions of the E1a 289R protein, it retains the ability of the 289R protein to bind to p107. Elevated levels of free E2F are the primary factor inducing the cell cycle. By retaining the p107 binding domain, the 289R protein will bind to a sequester p107 resulting in slightly elevated intracellular levels of E2F. Although this low level of E2F is insufficient to initiate cell cycle progression on its own in normal cells, in tumor cells, the ability of the 289R protein to bind up p107 produces an elevated level of E2F in excess of the E2F threshold level necessary to induce cell cycling thereby enhancing the ability of the virus to replicate. This results in enhanced cytotoxicity of the virus in tumor cells while not affecting the toxicity of the virus to normal cells.

The term "recombinant adenovirus" is synonymous with the term "recombinant adenoviral vector" and refers to viruses of the genus adenoviridiae capable to infecting a cell whose genomes have been modified through conventional recombinant DNA techniques. The term adenoviridiae refers collectively to animal adenoviruses of the genus mastadenovirus including but no limited to human, bovine, ovine, equine, canine, porcine, murine and simian adenovirus subgenera. In particular, human adenoviruses includes the A-F sugenera as well as the individual serotypes thereof the individual serotypes and A-F subgenera including but not limited to human adenovirus types 1, 2, 3, 4, 4a, 5, 6, 7, 8, 9, 10, 11 (Ad11A and Ad 11P), 12,

13,14,15,16,17,18,19, 19a, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 34a, 35, 35p, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, and 91. The term bovine adenoviruses includes but is not limited to bovine adenovirus types 1,2,3,4,7, and 10. The term canine adenoviruses includes but is not limited to canine types 1 (strains CLL, Glaxo, RI261, Utrect, Toronto 26-61) and 2. The term equine adenoviruses includes but is not limited to equine types 1 and 2. The term porcine adenoviruses includes but is not limited to porcine types 3 and 4. The term recombinant adenovirus also includes chimeric (or even multimeric) vectors, i.e. vectors constructed using complementary coding sequences from more than one viral subtype. See, e.g. Feng, *et al.* Nature Biotechnology 15:866-870. In the preferred embodiment of the invention, the adenovirus is a human adenovirus of serotype 2 or 5.

The term "modifications" refers to changes in the genomic structure of the recombinant adenoviral vector. Such modifications include deletions and/or changes in amino acid coding sequence so as to produce a protein deficient in binding to its substrate. For example, the Rb-105 binding domain of the E1a-12S and 13S proteins has been characterized as located within amino acids 111-127. The p300 binding domain of the E1a-12S and 13S proteins has been narrowed to the first 69 amino acids. Egan, *et al.* (1988) Mol. Cell Biol. 8:3955-3959. However, it has been shown that amino acids 26 to 35 are not necessary for p300 binding. There are two regions of p300 binding in the 12S and 13S molecules from approximately amino acids 4-25 and amino acids 36-49. Elimination of one or both is sufficient to disrupt p300 binding. Preferably, the elimination of amino acids in the 4-25 region are employed to eliminate the p300 binding function.

The term "deficient in binding" refers to a gene product forming a complex with less than 50% of the thermodynamic stability of the complex of the wild type gene product to its substrate under physiological conditions. For example, a 13S gene product which contains a deletion in the p300 binding domain would bind to p300 protein with less than 50% of the thermodynamic stability of the wild-type 13S protein. The thermodynamic stability of binding can readily be determined by conventional assay techniques to determine equilibrium binding constants under physiological conditions.

The term "E1a gene" refers to the immediate early gene of the adenovirus genome first transcribed following infection. This genomic sequence represents at least the transcription of five mRNAs encoding the 9S, 10S, 11S, 12S and 13S proteins. The 12S and 13S proteins are expressed in the early phase following infection while the 9S, 10S and 11S proteins are expressed later in the adenovirus cycle. The 12S and 13S proteins have 243 and 289 amino acids respectively. There are three conserved regions in the E1a genomic sequence referred to as conserved region ("CR")-1, CR2 and CR3. CR1 represents amino acids 41-80 of the 12S and 13S proteins. CR2 represents amino acids 121-139 of the 12S and 13S sequence.

The "transactivating function of the CR3 domain" refers to the ability of the products of the E1a gene to activate transcription of promoters later in the viral cycle such as E1b and E2. The CR3 region is functionally present only in the 13S protein and represents amino acids 140 to 188. The transactivating function of the E1a gene product is contained in the CR3 region. The transactivating' region is retained in the vectors of the present invention to permit activation of other viral genes and improve the cytotoxicity.

The term "p300 protein family members" refers the proteins which associate with the amino terminus of E1a including p300 and CBP. In particular p300 co-activates the activity of the transactivating genes, Myb and C/EBP. Mink, *et al.* (1997) Molecular and Cellular Biology 17:6609-6617. The human p300 protein is known in the art and is publicly available from the Swiss-Prot database under accession number Q09472, its corresponding mRNA is available from GenBank under accession number U01877 deposited June 6, 1994 and is described in Eckner, *et al.* (1994) Genes Dev. 8:869-884.

The term "Rb protein family members" refers to the retinoblastoma gene product (pb105), p107 and p130. The retinoblastoma gene is well characterized in the art. The amino acid sequence of human Rb is available from GenBank under accession Number 190959 deposited July 12, 1995 and the mRNA sequence is available from GenBank under accession number M15400 and is described in Lee, *et al.* (1988) PNAS (USA) 85:6017-6021.

In order to demonstrate the efficacy of the vectors of the present invention, the vectors were evaluated first using *in vitro* experiments. The *in vitro* experiments presented below were designed to determine the potential of the vectors to selectively destroy tumor cells as compared to wild type virus and several other vector constructions. The outcome of these experiments would determine the potential of the vectors as agents for treatment of hyperproliferative diseases including cancer. The vectors were prepared in substantial accordance with the teaching of Example 1 herein. The vectors used for comparison include: wild type adenovirus type 5 (Ad5wt), dl309 a phenotypic wild type Type 5 human adenovirus containing modifications to eliminate certain E3 functions (as described in Jones and Shenk (1978) Cell 13:181-188; and Jones and Shenk (1979) Cell 17:683-689); a mutant adenovirus (E1bdl55K) which contains a deletion of the majority of the E1bdl55K gene that eliminates production of a functional E1bdl55K protein prepared in substantial accordance with the teaching of Example 2 herein; mutant adenovirus E1bdl55K/309 which contains a double point mutation in the coding region of the E1b55K gene and therefore also eliminates production of the E1b55K protein (McLorie, *et al.* (1991) J. Gen. Virol. 72:1467-1471), the adenovirus based gene therapy vector ACN53 (Wills, *et al.* (1994) Human Gene Therapy 5:1079-1088) in which the complete E1 region is replaced with the p53 gene under control of the CMV promoter; and a control vector ACN which contains a complete E1 region deletion but does not encode a therapeutic transgene.

The ability of the vectors to destroy cells *in vitro* was evaluated in normal diploid fibroblasts and in several tumor lines by using an the CPE assay to evaluate the cell killing by the vector constructions. The CPE assay is described in Bischoff, *et al.* (1996) Science 274:373-376. This assay involves infecting cells in culture with a range of viral particle concentrations of the test viral constructs, and then staining with crystal violet the infected cells at time points after infection to determine the number of viable cells left on the cell culture growth substrate. Cell killing as a result of replication competent adenovirus infection results in cell lysis and detachment of the infected cells from the growth substrate. Therefore, the number of cells remaining can be used as a qualitative measure of the cell killing potential of the viral construct. Cell killing by the replication incompetent p53 gene delivery vector ACN53 can also be evaluated using

this assay. In contrast to the replication competent vectors, cell death as a result ACN53 occurs in many tumor cells as a result of p53-dependent apoptosis which also leads to detachment of cells from the growth matrix. Therefore it is possible to use the assay described above to compare cell death as a result of p53-induced apoptosis with cell killing by lysis with the replication competent vectors.

Representative results from experiments in which the normal fibroblast cell line MRC9 ($p53^+$, pRb^+), were infected with the test viral constructions are shown in Figure 4, 5 and 6. In MRC9 cells infected at a low concentration of $1.8X10^7$ particles/ml some cell detachment was observed only in wtAd5 infected MRC9 cells, but not with the other constructs. At an infection concentration of $1.8X10^8$ particles/m l wtAd5 infected MRC9 cells were efficiently destroyed and there was some evidence of killing in the E1Bdl55K infected cells. Infection at a high concentration of $1.8X10^9$ particles/ml resulted in almost complete cell killing in MRC9 cells infected with wtAd5 and E1bdl55K. Cell killing was also observed with E1Adl01/07 after infection at $1.8X10^8$ particles/ml, but at a reduced level compared to the E1bdl55K and wtAd5 vectors. The ACN53 vector for p53 gene delivery had only a minor toxic effect on the MRC9 cells, as compared to the empty vector control, and only at the high infection concentration of $1.8X10^9$ particles/ml.

The test vectors were next used to evaluate their potential for cell killing in the cervical carcinoma line C33A ($p53^-$, pRb^-). The results of representative experiments are shown in Figures 1, 2, and 3. After infection of the C33A cells at $1.8X10^9$ particles/ml all of the viral constructions, with the exception of the E1-deficient control vector ACN, efficiently destroyed the entire monolayer of cells. Infection with wtAd5 and E1Adl01/07 at concentrations of $1.8X10^8$ particles/ml and $1.8X10^7$ also resulted in complete destruction of the C33A monolayers. However, the E1Bdl55K vector was somewhat defective for cell killing after infection at $1.8X10^8$ particles/ml and significantly defective at $1.8X10^7$ particles/ml . The ACN53 vector for p53 gene delivery did not induce significant cell death beyond the high infection level of $1.8X10^9$ particles/ml as illustrated by the number of C33A cells remaining after infection at $1.8X10^8$ and $1.8X10^7$ particles/ml of this construct.

To further characterize the vectors for viral growth *in vitro* a panel of cells was infected with the test constructs and viral replication was determined quantitatively at 48 hours after infection using the procedure of Huyghe *et al.* (1995) *Human Gene Therapy* 6: 1403-1416. The cell lines evaluated in addition to the normal line MRC9 ($p53^+$, pRb^+) and the C33A ($p53^-$, pRb^-) tumor line were a lung non-small cell carcinoma line H358 ($p53^{null}$, pRb^+) and a liver carcinoma line SKHep1 ($p53^+$, pRb^+). The results of representative experiments in which cells were infected either at $1.8x10^9$ or $1.8x10^8$ particles/ml are shown in Figure 3. The results of these experiments show that E1bdl55K was defective for growth in all cell lines as compared to wtAd5, but was most defective in the MRC9 normal line, and the lung tumor line H358. The E1Adl01/07 construct was, as expected, was defective for virus production in the normal MRC9 cells, but replicated as efficiently as wild type virus and dl309 virus in the C33A cervical carcinoma line. In both the lung carcinoma H358 ($p53^{null}$, pRb^+) and the hepatocellular carcinoma SKHep1($p53^+$, pRb^+) cells the E1Adl01/07 virus replicated slightly less efficiently than wtAd5 and dl309, but the construct consistently replicated more efficiently than the E1Bdl55K virus.

Together the results of the above experiments showed that the E1Adl01/07 vector induced cell killing more efficiently in tumor cell lines than in normal cells, and that the status of the tumor suppressor genes pRb and p53 did not appear to affect the ability of the virus to replicate in tumor cells. In addition, our results differ from those of Bischoff, *et al.* (1996) Science 274:373-376, who reported that an E1bdl55K vector replicated as efficiently as wtAd5 in p53-defective cell lines, including the C33A carcinoma cell line studied here, but was replication defective in p53+ cells lines. See Bischoff, *et al.* (1996) Science 274:373-376. Our results show that E1Bd55K constructs are defective for cell growth in both normal and tumor cell lines.

In order to demonstrate the efficacy of the vectors of the present invention, the vectors of the present invention were evaluated in a *in vivo* model of cervical carcinoma. The mouse model was based on the establishment of tumors and then treated with the above vectors in substantial accordance with the teaching of Example 3 herein. The results are shown in Table 2 provided below.

Table 2.

Tumor Volume (mm^3) In Response to Treatment With rAd Vectors

Vector	D6	D9	D13	D16	D20	D23	D27	D30	D34
vPBS	128	128	380	463	808	1215	2116	2583	4153
dl309	112	112	115	91	75	56	52	73	78
E1Adl01/07	114	114	95	70	53	43	45	57	60
E1Bdl55k	102	102	114	114	92	78	153	221	299
ACN53	118	118	143	163	229	353	657	1016	1297

As can be seen from the data presented in Table 2, the vectors of the present invention as exemplified by E1Adl01/07, demonstrate effective *in vivo* anti-tumor activity. Additionally, it should be noted that the double deleted E1Adl01/07 vector achieved a reduction in tumor size approximately equivalent to the wild-type dl309 virus. This is in substantial contrast to the ACN53 and E1Bdl55K vectors which merely slowed the growth of the tumor.

Additional in vivo studies were performed in nude mice to evaluate the efficacy of the vectors of the present invention following both intratumoral and intravenous systemic administration of virus. The first study was DLD-1 tumor model to assess the efficacy of the intratumorally administered dl01/07 virus. The results of the study are presented in Figure 13 of the accompanying drawings. As can be seen from the data presented, the dl01/07/309 virus was effective in preventing tumor growth at least as (if not more) effectively than the wild-type virus and significantly better than the E1bdl55K virus. These results are particularly significant as the DLD-1 tumors are very aggressive. The second model was designed to assess the efficacy of the dl01/07 virus when administered systemically by intravenous injection. The results of this study are presented in Figure 14 of the accompanying drawings. As can be seen from the data presented, the dl01/07 virus is effective in reducing tumor growth in vivo following systemic administration. The fact that the virus was able to find the tumor following systemic administration and slow its growth in the absence of any targeting

modifications to the virus is particularly relevant. Together, these studies demonstrate that selectively replicating viruses containing deletions of 289R p300 and pRb105 binding functions are useful in the treatment of tumors when administered either intratumorally or systemically.

In the preferred practice of the invention, the recombinant adenoviral vector is derived from genus adenoviridiae. Particularly preferred viruses are derived from the human adenovirus type 2 or type 5. In the preferred practice of the invention, the vector is derived from the human adenoviridiae. More preferred are vectors derived from human adenovirus subgroup C. Most preferred are adenoviral vectors derived from the human adenovirus serotypes 2 and 5. In the most preferred practice of the invention the virus is derived from human adenovirus Type 5 dl309 or dl520.

The vectors of the present contain deletions in the E1a coding sequence to eliminate p300 and p105-Rb binding sites in the 13S coding sequence. In the preferred practice of the invention, the p300 binding deletions are represented by deletions of amino acids from about 4 to about 25 or from about 36 to about 49. In the preferred practice of the invention, the Rb binding deletions are represented by elimination of amino acids from about 111-127, preferably from about 111-123. More preferred is a vector wherein said deletion in the E1a-p300 binding domain comprises a deletion of the codons for amino acids 4 to 25 of the adenoviral E1a gene product. More preferred is a vector wherein deletion in the E1a-Rb binding domain comprises a deletion of the codons for amino acids 111-123 of the adenoviral E1a gene product. Alternatively, pRb binding may be eliminated by the introduction of a mutation to eliminate amino acids 124-127 of the E1A 289R and 243R proteins. In the most preferred embodiment of the present invention as exemplified herein the vector comprises a deletion of amino acids 4-25 and 111-123 of the E1a 13S gene product.

The invention further provides a recombinant adenovirus which contains modifications to the E1a coding sequence so as to produce E1a gene products which are deficient in binding to one or more p300 protein family members and one or more Rb protein family member protein but retain the transactivating function of the E1a CR3 domain and a deletion of the amino acids from approximately 219 to approximately 289 of the E1a 289R protein (or approximately amino acids 173 to approximately amino

acid 243 of the E1a 243R protein. In the preferred practice of the invention the deletion of the binding to the p300 family members is achieved by introducing a deletion corresponding to amino acids 4-25 of the E1a 243R and 289R proteins or amino acids 38-60 of the E1a 243R and 289R proteins. In the preferred practice of the invention the deletion of the binding to the pRb family members is achieved amino acids 111-123 of the E1a 243R and 289R proteins. Alternatively, deletion of the binding to the pRb family members may be achieved by eliminate of amino acids 124-127 of the E1a 243R and 289R proteins.

As previously described, the deletions in the adenoviral genome result in preferential replication in rapidly dividing cells. These desirable features may be combined with other elements to provide even greater degrees of selectivity and/or cytotoxicity to such cells. For example, the vectors of the present invention also include recombinant adenoviruses containing modifications to the viral genome to induce preferential replication in particular cell types using cell type specific promoters or inducible promoters. The term "cell type specific promoter" refers to promoters which are differentially activated in as a result of cell cycle progression or in different cell types. Examples of cell-type specific promoters includes cell cycle regulatory gene promoters, tissue specific of tumor specific promoters or pathway responsive promoters. The term "cell cycle regulatory gene promoters" describe promoters for genes which are activated substantially upon entry into S-phase. Examples of such promoters include the E2F regulated promoters (e.g. DHFR, DNA polymerase alpha, thymidylate synthase, c-myc and b-myb promoters). Tissue specific and tumor specific promoters are well known in the art and include promoters active preferentially in smooth muscle (α-actin promoter), pancreas specific (Palmiter, *et al.*(1987) Cell 50:435), liver specific Rovet, et.al. (1992) J. Biol. Chem.. 267:20765; Lemaigne, *et al.* (1993) J. Biol. Chem.. 268:19896; Nitsch, *et al.* (1993) Mol. Cell. Biol. 13:4494), stomach specific (Kovarik, *et al.* (1993) J. Biol. Chem.. 268:9917, pituitary specific (Rhodes, *et al.* (1993) Genes Dev. 7:913, prostate specific (United States Patent 5,698,443, Henderson, *et al.*issued December 16, 1997), etc.

The term "pathway-responsive promoter" refers to DNA sequences that bind a certain protein and cause nearby genes to respond transcriptionally to the binding of the

protein in normal cells. Such promoters may be generated by incorporating response elements which are sequences to which transcription factors bind. Such responses are generally inductive, though there are several cases where increasing protein levels decrease transcription. Pathway-responsive promoters may be naturally occurring or synthetic. Pathway-responsive promoters are typically constructed in reference to the pathway or a functional protein which is targeted. For example, a naturally occurring p53 pathway-responsive promoter would include transcriptional control elements activated by the presence of functional p53 such as the p21 or bax promoter. Alternatively, synthetic promoters containing p53 binding sites upstream of a minimal promoter (e.g. the SV40 TATA box region) may be employed to create a synthetic pathway-responsive promoter. Synthetic pathway-responsive promoters are generally constructed from one or more copies of a sequence that matches a consensus binding motif. Such consensus DNA binding motifs can readily be determined. Such consensus sequences are generally arranged as a direct or head-to-tail repeat separated by a few base pairs. Elements that include head-to-head repeats (e.g. AGGTCATGACCT) are called palindromes or inverted repeats and those with tail-to-tail repeats are called everted repeats.

Examples of pathway-responsive promoters useful in the practice of the present invention include synthetic insulin pathway-responsive promoters containing the consensus insulin binding sequence (Jacob, *et al.* (1995). J. Biol. Chem. 270:27773-27779), the cytokine pathway-responsive promoter, the glucocorticoid pathway-responsive promoter (Lange, *et al.*(1992) J Biol. Chem. 267:15673-80), IL1 and IL6 pathway-responsive promoters (Won K.-A and Baumann H. (1990) Mol.Cell.Biol. 10: 3965-3978), T3 pathway-responsive promoters, thyroid hormone pathway-responsive promoters containing the consensus motif: 5' AGGTCA 3.', the TPA pathway-responsive promoters (TREs), TGF–β pathway-responsive promoters (as described in Grotendorst, *et al.*(1996) Cell Growth and Differentiation 7: 469-480). Additionally, natural or synthetic E2F pathway responsive promoters may be used. An example of an E2F pathway responsive promoter is described in Parr, *et al.* (1997, Nature Medicine 3:1145-1149) which describes an E2F-1 promoter containing 4 E2F binding sites and is reportedly active in tumor cells with rapid cycling. Examples of other

pathway-responsive promoters are well known in the art and can be identified in the Database of Transcription Regulatory Regions on Eukaryotic Genomes accessible through the internet at http://www.eimb.rssi.ru/TRRD.

In the preferred practice of the invention as exemplified herein, the vector comprises a synthetic TGF–β pathway-responsive promoter active in the presence of a functional TGF–β pathway such as the promoter containing SRE and PAI pathway responsive promoters. PAI refers to a synthetic TGF–β pathway-responsive promoter comprising sequences responsive to TGF–β signally isolated from the plasminogen activator-I promoter region. The PAI-pathway-responsive promoter may be isolated as a 749 base pair fragment isolatable from the plasmid p800luc (as described in Zonneveld, *et al.* (1988) PNAS 85:5525-5529 and available from GenBank under accession number J03836). SRE refers to a synthetic TGF–β response element comprising a repeat of 4 of the Smad-4 DNA binding sequences (GTCTAGAC as described in Zawel, *et al.* (1988) Mol. Cell 1:611-617. The SRE response element may be generated by annealing complimentary oligonucleotides encoding the Smad-4 binding sequences and cloning in plasmid pGL#3 - promoter luciferase vector (commercially available from ProMega).

Similarly, a "p53 pathway-responsive promoter" refers to a transcriptional control element active in the presence of a functional p53 pathway. The p53 pathway-responsive promoter may be a naturally occurring transcriptional control region active in the presence of a functional p53 pathway such as the p21 or mdm2 promoter. Alternatively, the p53 pathway-responsive promoter may be a synthetic transcriptional control region active in the presence of a functional p53 pathway such as the SRE and PAI-RE pathway-responsive promoters. p53-CON describes a p53 pathway-responsive promoter containing a synthetic p53 response element constructed by insertion of two synthetic p53 consensus DNA binding sequences (as described in Funk, *et al.* (1992) Mol.Cell Biol. 12:2866-2871) upstream of the SV40 TATA box. RGC refers to a synthetic p53 pathway-responsive promoter using a tandem of the p53 binding domains identified in the ribosomal gene cluster. p53CON and RGC response elements can be constructed by annealing complementary oligonucleotides and p53

responsive promoters can be constructed by cloning in plasmid pGL3-promoter luciferase vector (commercially available from ProMega)

Alternatively, the viral genome may be modified to include inducible promoters which are functional under certain conditions in response to chemical or other stimuli. Examples of inducible promoters are known in the scientific literature (See, e.g. Yoshida and Hamada (1997) Biochem. Biophys. Res. Comm. 230:426-430; Iida, *et al.* (1996) J. Virol. 70(9):6054-6059; Hwang, *et al.* (1997) J. Virol 71(9):7128-7131; Lee, *et al.* (1997) Mol. Cell. Biol. 17(9):5097-5105; and Dreher, *et al.* (1997) J. Biol. Chem. 272(46); 29364-29371. An example of radiation inducible promoters include the EGR-1 promoter. Boothman, *et al.* (1994) volume 138 supplement pages S68-S71

The cell type specific (tissue specific, tumor specific, pathway specific, cell cycle regulatory promoter) promoter or inducible promoter may be used in lieu of the native E1a promoter region in the vectors of the present invention to provide preferential expression in particular cell types.

Alternatively, the one may use a pathway responsive promoter to drive expression of a repressor of viral replication. The term "repressor of viral replication" refers to a protein, if expressed in a given cell, substantially represses viral replication. In the case of adenoviral vectors, the E2F-Rb fusion construct as described in European Patent Application No. 94108445.1 published December 6, 1995 (Publication number. 0 685 493 A1) may be employed. E2F-Rb fusion protein consists of the DNA binding and DP1 heterodimerization domains of the human E2F-2 transcription factor protein (amino acids 95-286 of wild type E2F) fused to the Rb growth suppression domain (amino acids 379-928 of the wild type Rb protein). The E2F-Rb fusion protein is a potent repressor of E2F transcription and arrests cells in G1. The DNA binding domain is located at amino acids 128-193 and the dimerization domain is located at 194-289. The sequence of the human E2F2 protein is available from GenBank under accession number 2494288 deposited November 1, 1997 as updated July 15, 1998.

These modifications may be combined with the previously cell cycle regulatory gene promoters described above. For instance, an E2F pathway responsive promoter may be used to drive expression of the modified E1a coding sequence. Using a p53 pathway responsive promoter driving expression of E2F-Rb fusion protein, one

achieves repression of both E1 function and E2 function because the E2F-Rb fusion protein will suppress both the E2 and E2F response elements. In p53 deficient tumor cells, the p53 response element is inactive and E2F-Rb is not expressed. Consequently, E1a expression is enhanced by the presence of E2F in the tumor cell and the failure to repress E2 promoter enables viral replication to proceed.

As previously described, the deletions in the adenoviral genome result in preferential replication in rapidly dividing cells. While these viruses will replicate and ultimately kill the tumor cells, these viruses may also incorporate a therapeutic transgene expression cassette to enhance cytotoxicity. The term "expression cassette" is used herein to define a nucleotide sequence containing regulatory elements and a transgene coding sequence so as to result in the transcription and translation of a transgene in a transduced cell. The term "regulatory element" refers to promoters, enhancers, transcription terminators, polyadenylation sites, and the like. The regulatory elements may be arranged so as to allow, enhance or facilitate expression of the transgene only in a particular cell type. For example, the expression cassette may be designed so that the transgene is under control of an inducible promoter, tissue specific or tumor specific promoter, or temporal promoter. The term "temporal promoters" refers to promoters which drive transcription or the therapeutic transgene at a point later in the viral cycle relative to the promoter controlling expression of the response element and are used in conjunction with viral vector systems. Examples of such temporally regulated promoters include the adenovirus major late promoter (MLP), other late promoters.

The term "therapeutic transgene" refers to a nucleotide sequence the expression of which in the target cell produces a cytotoxic or cytostatic effect. The term therapeutic transgene includes but is not limited to tumor suppressor genes, antigenic genes, cytotoxic genes, dendritic cell chemoattractants, cytostatic genes, pro-drug activating genes, or pro-apoptotic. The vectors of the present invention may be used to produce one or more therapeutic transgenes, either in tandem through the use of IRES elements or through independently regulated promoters.

The term "tumor suppressor gene" refers to a nucleotide sequence, the expression of which in the target cell is capable of suppressing the neoplastic phenotype and/or inducing apoptosis. Examples of tumor suppressor genes useful in the practice

of the present invention include the p53 gene, the APC gene, the DPC-4 gene, the BRCA-1 gene, the BRCA-2 gene, the WT-1 gene, the retinoblastoma gene (Lee, *et al.* (1987) Nature 329:642), the MMAC-1 gene, the adenomatous polyposis coli protein (Albertsen, *et al.*, United States Patent 5,783,666 issued July 21, 1998), the deleted in colon carcinoma (DCC) gene, the MMSC-2 gene, the NF-1 gene, nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3. (Cheng, *et al.* 1998. Proc. Nat. Acad. Sci. 95:3042-3047), the MTS1 gene, the CDK4 gene, the NF-1 gene, the NF2 gene, and the VHL gene.

The term "antigenic genes" refers to a nucleotide sequence, the expression of which in the target cells results in the production of a cell surface antigenic protein capable of recognition by the immune system. Examples of antigenic genes include carcinoembryonic antigen (CEA), p53 (as described in Levine, A. PCT International Publication No. WO94/02167 published February 3, 1994). In order to facilitate immune recognition, the antigenic gene may be fused to the MHC class I antigen.

The term "cytotoxic gene" refers to nucleotide sequence, the expression of which in a cell produces a toxic effect. Examples of such cytotoxic genes include nucleotide sequences encoding pseudomonas exotoxin, ricin toxin, diptheria toxin, and the like.

The term "cytostatic gene" refers to nucleotide sequence, the expression of which in a cell produces an arrest in the cell cycle. Examples of such cytostatic genes include p21, the retinoblastoma gene, the E2F-Rb gene, genes encoding cyclin dependent kinase inhibitors such as P16, p15, p18 and p19, the growth arrest specific homeobox (GAX) gene as described in Branellec, *et al.* (PCT Publication WO97/16459 published May 9, 1997 and PCT Publication WO96/30385 published October 3, 1996).

The term "dendritic cell chemoattractants" refers to chemotactic chemokines capable of attracting and/or directing the migration of dendritic cells to a particular location. It has been demonstrated that certain chemokines, fMLP (representative of formyl peptides of bacterial origin), C5a and the C-C chemokines monocyte chemotactic protein (MCP)-3, macrophage inflammatory protein (MIP)-1 α/LD78, and RANTES, have been involved in the recruitment and chemotactic migration of dendritic cells. Sozzani, *et al.* (1995) J. Immunol. 1995 155(7):3292-5. Xu, *et al.* suggest that

all C-C chemokines, including MCP-1, MCP-2, MCP-3, MIP1 α, MIP-1 β, and RANTES, induced migration of DC-enriched cells cultured with or without IL-4. Xu, *et al.*. (1996) J. Leukoc. Biol. 60(3):365-71. Greaves, *et al.* (1997) J. Exp. Med. 186(6):837-44, indicate that MIP-3-α specifically interacts with the CC chemokine receptor 6 expressed on dendritic cells capable of directing migration of dendritic cells. In the preferred practice of the invention, the dendritic cell chemoattractant is MIP-3-α. The dendritic cell chemoattractant may be expressed intracellular form where it is released upon cell lysis or in secreted form by the use of a signal peptide.

The term "pro-apoptotic gene" refers to a nucleotide sequence, the expression thereof results in the programmed cell death of the cell. Examples of pro-apoptotic genes include p53, adenovirus E3-11.6K, the adenovirus E4orf4 gene, p53 pathway genes, and genes encoding the caspases.

The term "pro-drug activating genes" refers to nucleotide sequences, the expression of which, results in the production of protein capable of converting a non-therapeutic compound into a therapeutic compound, which renders the cell susceptible to killing by external factors or causes a toxic condition in the cell. An example of a prodrug activating gene is the cytosine deaminase gene. Cytosine deaminase converts 5-fluorocytosine to 5 fluorouracil, a potent antitumor agent). The lysis of the tumor cell provides a localized burst of cytosine deaminase capable of converting 5FC to 5FU at the localized point of the tumor resulting in the killing of many surrounding tumor cells. This results in the killing of a large number of tumor cells without the necessity of infecting these cells with an adenovirus (the so-called bystander effect"). Additionally, the thymidine kinase (TK) gene (see e.g. Woo, *et al.* United States Patent No. 5,631,236 issued May 20, 1997 and Freeman, *et al.* United States Patent No. 5,601,818 issued February 11, 1997) in which the cells expressing the TK gene product are susceptible to selective killing by the administration of gancyclovir may be employed.

It will be readily apparent to those of skill in the art that modifications and or deletions to the above referenced genes so as to encode functional subfragments of the wild type protein may be readily adapted for use in the practice of the present invention. For example, the reference to the p53 gene includes not only the wild type protein but

also modified p53 proteins. Examples of such modified p53 proteins include modifications to p53 to increase nuclear retention, deletions such as the Δ13-19 amino acids to eliminate the calpain consensus cleavage site, modifications to the oligomerization domains (as described in Bracco, *et al.* PCT published application WO97/0492 or United States Patent No. 5,573,925).

It will be readily apparent to those of skill in the art that the above therapeutic genes may be secreted into the media or localized to particular intracellular locations by inclusion of a targeting moiety such as a signal peptide or nuclear localization signal(NLS). Also included in the definition of therapeutic transgene are fusion proteins of the therapeutic transgene with the herpes simplex virus type 1 (HSV-1) structural protein, VP22. Fusion proteins containing the VP22 signal, when synthesized in an infected cell, are exported out of the infected cell and efficiently enter surrounding non-infected cells to a diameter of approximately 16 cells wide. This system is particularly useful in conjunction with transcriptionally active proteins (e.g. p53) as the fusion proteins are efficiently transported to the nuclei of the surrounding cells. See, e.g. Elliott, G. & O'Hare, P. Cell. 88:?23-233:1997; Marshall, A. & Castellino, A. Research News Briefs. Nature Biotechnology. 15:205:1997; O'Hare, *et al.* PCT publication WO97/05265 published February 13, 1997. A similar targeting moiety derived from the HIV Tat protein is also described in Vives, *et al.* (1997) J. Biol. Chem. 272:16010-16017.

The present invention provides recombinant adenoviruses which contain "targeting modifications" in order to achieve preferential targeting of the virus to a particular cell type. The term "targeting modification" refers to modifications to the viral genome designed to result in preferential infectivity of a particular cell type. Cell type specificity or cell type targeting may also be achieved in vectors derived from viruses having characteristically broad infectivities such as adenovirus by the modification of the viral envelope proteins. For example, cell targeting has been achieved with adenovirus vectors by selective modification of the viral genome knob and fiber coding sequences to achieve expression of modified knob and fiber domains having specific interaction with unique cell surface receptors. Examples of such modifications are described in Wickham, *et al.* (1997) J. Virol 71(11):8221-8229

(incorporation of RGD peptides into adenoviral fiber proteins); Arnberg, *et al.* (1997) Virology 227:239-244 (modification of adenoviral fiber genes to achieve tropism to the eye and genital tract); Harris and Lemoine (1996) TIG 12(10):400-405; Stevenson, *et al.* (1997) J. Virol. 71(6):4782-4790; Michael, *et al.*(1995) gene therapy 2:660-668 (incorporation of gastrin releasing peptide fragment into adenovirus fiber protein); and Ohno, *et al.* (1997) Nature Biotechnology 15:763-767 (incorporation of Protein A-IgG binding domain into Sindbis virus). Other methods of cell specific targeting have been achieved by the conjugation of antibodies or antibody fragments to the envelope proteins (see, e.g. Michael, *et al.* (1993) J. Biol. Chem. 268:6866-6869, Watkins, *et al.* (1997) gene therapy 4:1004-1012; Douglas, *et al.* (1996) Nature Biotechnology 14: 1574-1578. Alternatively, particular moieties may be conjugated to the viral surface to achieve targeting (See, e.g. Nilson, *et al.* (1996) gene therapy 3:280-286 (conjugation of EGF to retroviral proteins). These recombinantly modified vectors may be produced in accordance with the practice of the present invention.

The present invention further provides recombinant adenoviral vectors comprising a suicide gene. In some instances, it may be desirable to include a suicide gene in the viral vector. This provides a "safety valve" to the viral vector delivery system to prevent widespread infection due to the spontaneous generation of replication competent viral vectors. The term "suicide gene" refers to a nucleic acid sequence, the expression of which renders the cell susceptible to killing by external factors or causes a toxic condition in the cell. A well known example of a suicide gene is the thymidine kinase (TK) gene (see e.g. Woo, *et al.* United States Patent No. 5,631,236 issued May 20, 1997 and Freeman, *et al.* United States Patent No. 5,601,818 issued February 11, 1997) in which the cells expressing the TK gene product are susceptible to selective killing by the administration of gancyclovir.

The present invention further provides a pharmaceutically acceptable formulation of the recombinant adenoviruses in combination with a carrier. The vectors of the present invention may be formulated for dose administration in accordance with conventional pharmaceutical practice with the addition of carriers and excipients. Dosage formulations may include intravenous, intratumoral, intramuscular, intraperitoneal, topical, matrix or aerosol delivery.

The term "carrier" refers to compounds commonly used on the formulation of pharmaceutical compounds used to enhance stability, sterility and deliverability of the therapeutic compound. When the virus is formulated as a solution or suspension, the delivery system is in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorption monolaurate, triethanolamine oleate, etc.

The present invention further provides pharmaceutical formulations of the vectors of recombinant adenoviruses of the present invention with a carrier and a delivery enhancing agent(s). The terms delivery enhancers" or "delivery enhancing agents" are used interchangeably herein and includes one or more agents which facilitate uptake of the virus into the target cell. Examples of delivery enhancers are described in co-pending United States Patent Application Serial No. _____ filed July 7, 1998. Examples of such delivery enhancing agents include detergents, alcohols, glycols, surfactants, bile salts, heparin antagonists, cyclooxygenase inhibitors, hypertonic salt solutions, and acetates. Alcohols include for example the aliphatic alcohols such as ethanol, N-propanol, isopropanol, butyl alcohol, acetyl alcohol. Glycols include glycerine, propyleneglycol, polyethyleneglycol and other low molecular weight glycols such as glycerol and thioglycerol. Acetates such as acetic acid, gluconic acid, and sodium acetate are further examples of delivery-enhancing agents. Hypertonic salt solutions like 1M NaCl are also examples of delivery-enhancing agents. Examples of surfactants are sodium dodecyl sulfate (SDS) and lysolecithin, polysorbate 80, nonylphenoxypolyoxyethylene, lysophosphatidylcholine, polyethyleneglycol 400, polysorbate 80, polyoxyethylene ethers, polyglycol ether surfactants and DMSO. Bile

salts such as taurocholate, sodium tauro-deoxycholate, deoxycholate, chenodesoxycholate, glycocholic acid, glycochenodeoxycholic acid and other astringents such as silver nitrate may be used. Heparin-antagonists like quaternary amines such as protamine sulfate may also be used. Cyclooxygenase inhibitors such as sodium salicylate, salicylic acid, and non-steroidal antiinflammatory drug (NSAIDS) like indomethacin, naproxen, diclofenac may be used.

The term "detergent" includes anionic, cationic, zwitterionic, and nonionic detergents. Exemplary detergents include but are not limited to taurocholate, deoxycholate, taurodeoxycholate, cetylpyridium, benalkonium chloride, Zwittergent3-14 detergent, CHAPS (3-[(3-Cholamidopropyl) dimethylammoniol]-1-propanesulfonate hydrate), Big CHAP, Deoxy Big CHAP, Triton-X-100 detergent, C12E8, Octyl-B-D-Glucopyranoside, PLURONIC- F68 detergent, Tween 20 detergent, and TWEEN 80 detergent (CalBiochem Biochemicals).

Unit dosage formulations of the present invention may be included in a kit of products containing the recombinant adenovirus of claim 1 in lyophilized form and a solution for reconstitution of the lyophilized product. Recombinant adenoviruses of the present invention may be lyophilized by conventional procedures and reconstituted.

The present invention provides a method of ablating neoplastic cells in a mammalian organism *in vivo* by the administration of a pharmaceutically acceptable formulation of the recombinant adenovirus of the present invention. The term "ablating" means the substantial reduction of the population of viable neoplastic cells so as to alleviate the physiological maladictions of the presence of the neoplastic cells. The term "substantial" means a reduction in the population of viable neoplastic cells in the mammalian organism by greater than approximately 20% of the pretreatment population. The term "viable" means having the uncontrolled growth and cell cycle regulatory characteristics of a neoplastic cell. The term "viable neoplastic cell" is used herein to distinguish said cells from neoplastic cells which are no longer capable of replication. For example, a tumor mass may remain following treatment, however the population of cells comprising the tumor mass may be dead. These dead cells have been ablated and lack the ability to replicate, even though some tumor mass may remain.

The term "mammalian organism" includes, but is not limited to, humans, pigs, horses, cattle, dogs, cats. Preferably one employs an adenoviral vector endogenous to the mammalian type being treated. Although it is generally favored to employ a virus from the species to be treated, in some instances it may be advantageous to use vectors derived from different species which possess favorable pathogenic features. For example, it is reported (WO 97/06826 published April 10, 1997) that ovine adenoviral vectors may be used in human gene therapy to minimize the immune response characteristic of human adenoviral vectors. By minimizing the immune response, rapid systemic clearance of the vector is avoided resulting in a greater duration of action of the vector.

While the present invention provides a method of use of the recombinant adenoviruses alone, the recombinant adenoviruses of the present invention and formulations thereof may be employed in combination with conventional chemotherapeutic agents or treatment regimens. Examples of such chemotherapeutic agents include inhibitors of purine synthesis (e.g., pentostatin, 6-mercaptopurine, 6-thioguanine, methotrexate) or pyrimidine synthesis (e.g. Pala, azarbine), the conversion of ribonucleotides to deoxyribonucleotides (e.g. hydroxyurea), inhibitors of dTMP synthesis (5-fluorouracil), DNA damaging agents (e.g. radiation, bleomycines, etoposide, teniposide, dactinomycine, daunorubicin, doxorubicin, mitoxantrone, alkylating agents, mitomycin, cisplatin, procarbazine) as well as inhibitors of microtubule function (e.g vinca alkaloids and colchicine). Chemotherapeutic treatment regimens refers primarily to non-chemical procedures designed to ablate neoplastic cells such as radiation therapy. Examples of combination therapy when the therapeutic gene is p53 are described in Nielsen, *et al.* WO/9835554A2 published August 20, 1998.

The immunological response is significant to repeated administration of viral vectors. Consequently, the vectors of the present invention may be administered in combination with immunosuppressive agents. Examples of immunosuppressive agents include cyclosporine, azathioprine, methotrexate, cyclophosphamide, lymphocyte immune globulin, antibodies against the CD3 complex, adrenocorticosteroids, sulfasalzaine, FK-506, methoxsalen, and thalidomide.

The present invention also provides a method of ablating neoplastic cells in a population of normal cells contaminated by said neoplastic cells *ex vivo* by the administration of a recombinant adenovirus of the present invention to said population. An example of the application of such a method is currently employed in *ex vivo* applications such as the purging of autologous stem cell products commonly known as bone marrow purging. The term "stem cell product" refers to a population of hematopoietic, progenitor and stem cells capable of reconstituting the long term hematpoietic function of a patient who has received myoablative therapy. Stem cell products are conventionally obtained by apheresis of mobilized or non-mobilized peripheral blood. Apheresis is conventionally achieved through the use of known procedures using commercially available apheresis apparatus such as the COBE Spectra Apheresis System, commercially available from COBE International, 1185 Oak Street, Lakewood, CO. It is preferred that treatment conditions be optimized to achieve a "3-log purge" (i.e. removal of approximately 99.9% of the tumor cells from the stem cell produce) and most preferably a "5-log purge" (removal of approximately 99.999% of tumor cells from the stem cell product). In the preferred practice of the invention, a stem cell product of 100 ml volume would be treated with 1×10^5 to 1×10^9 particles of the recombinant adenovirus of the present invention for a period of approximately 4 hours at 37C.

In addition to therapeutic applications described above, the vectors of the present invention are also useful for diagnostic purposes. For example, the vectors of the present invention may incorporate a reporter gene which is expressed upon viral replication. The term "reporter gene" refers to a gene whose product is capable of producing a detectable signal alone or in combination with additional elements. Examples of reporter genes includes the β-galactosidase gene, the luciferase gene, the green fluorescent protein gene, nucleotide sequences encoding proteins detectable by imaging systems such as X-rays or magnetic field imaging systems (MRI). Alternatively, such vectors may also be employed to express a cell surface protein capable of recognition by a binding molecule such as a fluoroescently labeled antibody. Alternatively where the response element is used to drive a repressor of viral replication (e.g. E2F-Rb) later viral promoters (for example E2 which is turned off by E2F-Rb)

could be used to drive the reporter gene for diagnostic applications where the response element is off. These diagnostic constructs may be used for diagnostic purposes *in vivo* or *in vitro*. Examples of *in vivo* applications include imaging applications such as X-ray, CT scans or Magnetic Resonance Imaging (MRI).

The present invention further provides a method of producing the recombinant adenovirus comprising the modifications to the E1a gene product domains described above, said method comprising the steps of: :

a. infecting a producer cell with a recombinant adenovirus of the present invention,

b. culturing said infected producer cell under conditions so as to permit replication of the viral genome in the producer cell,

c. harvesting the producer cells, and

d. purifying the recombinant adenovirus.

The term "infecting" means exposing the recombinant adenovirus to the producer cell under conditions so as to facilitate the infection of the producer cell with the recombinant adenovirus. In cells which have been infected by multiple copies of a given virus, the activities necessary for viral replication and virion packaging are cooperative. Thus, it is preferred that conditions be adjusted such that there is a significant probability that the producer cells are multiply infected with the virus. An example of a condition which enhances the production of virus in the producer cell is an increased virus concentration in the infection phase. However, it is possible that the total number of viral infections per producer cell can be overdone, resulting in toxic effects to the cell. Consequently, one should strive to maintain the infections in the virus concentration in the range of 10^6 to 10^{10}, preferably about 10^9, virions per ml. Chemical agents may also be employed to increase the infectivity of the producer cell line. For example, the present invention provides a method to increase the infectivity of producer cell lines for viral infectivity by the inclusion of a calpain inhibitor. Examples of calpain inhibitors useful in the practice of the present invention include calpain inhibitor 1 (also known as N-acetyl-leucyl-leucyl-norleucinal, commercially available from Boehringer Mannheim). Calpain inhibitor 1 has been observed to increase the infectivity of producer cell lines to recombinant adenovirus.

The term "producer cell" means a cell capable of facilitating the replication of the viral genome of the recombinant adenovirus to be produced. A variety of mammalian cell lines are publicly available for the culture of recombinant adenoviruses. For example, the 293 cell line (Graham and Smiley (1977) J. Gen. Virol. 36:59-72) has been engineered to complement the deficiencies in E1a function and is a preferred cell line for the production of the current vectors. Examples of other producer cells include A549 cells, HeLa cells, PERC.6 cells (as described in publication WO/97/00326, application serial No. PCT/NL96/00244.

The term "culturing under conditions to permit replication of the viral genome" means maintaining the conditions for the infected producer cell so as to permit the virus to propagate in the producer cell. It is desirable to control conditions so as to maximize the number of viral particles produced by each cell. Consequently it will be necessary to monitor and control reaction conditions such as temperature, dissolved oxygen, pH, etc. Commercially available bioreactors such as the CelliGen Plus Bioreactor (commercially available from New Brunswick Scientific, Inc. 44 Talmadge Road, Edison, NJ) have provisions for monitoring and maintaining such parameters. Optimization of infection and culture conditions will vary somewhat, however, conditions for the efficient replication and production of virus may be achieved by those of skill in the art taking into considerations the known properties of the producer cell line, properties of the virus, type of bioreactor, etc. When 293 cells are employed as the producer cell line, oxygen concentration is preferably maintained from approximately 50% to approximately 120% dissolved oxygen, preferably 100% dissolved oxygen. When the concentration of viral particles (as determined by conventional methods such as HPLC using a Resource Q column) begins to plateau, the reactor is harvested.

The term "harvesting" means the collection of the cells containing the recombinant adenovirus from the media. This may be achieved by conventional methods such as diffential centrifugation or chromatographic means. At this stage, the harvested cells may be stored or further processed by lysis and purification to isolate the recombinant virus. For storage, the harvested cells should be buffered at or about physiological pH and frozen at -70C.

The term "lysis" refers to the rupture of the producer cells. Lysis may be achieved by a variety of means well known in the art. When it is desired to isolate the viral particles from the producer cells, the cells are lysed, using a variety of means well known in the art. For example, mammalian cells may be lysed under low pressure (100-200 psi differential pressure) conditions or conventional freeze thaw methods. Exogenous free DNA/RNA is removed by degradation with DNAse/RNAse.

The term "purifying" means the isolation of a substantially pure population of recombinant virus particles from the lysed producer cells. Conventional purification techniques such as chromatographic or differential density gradient centrifugation methods may be employed. In the preferred practice of the invention, the virus is purified by column chromatography in substantial accordance with the process of Huyghe *et al.* (1995) *Human Gene Therapy* 6: 1403-1416 as described in Shabram, et al., United States Patent 5,837,520 issued November 17, 1998, the entire teaching of which is herein incorporated by reference.

Additional methods and procedures to optimize production of the recombinant adenoviruses of the present invention are described in co-pending United States Patent Application Serial No. 09/073,076, filed May 4, 1998.

EXAMPLES

It will be apparent to those of skill in the art to which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed below without departing from the spirit or essential characteristics of the invention. The particular embodiments of the invention described below, are therefore to be considered as illustrative and not restrictive. In the following examples, "g" means grams, "ml" means milliliters, "mol" means moles, "°C" means degrees Centigrade, "min." means minutes, "FBS" means fetal bovine serum, and "PN" specifies particle number.

Example 1. Construction of Recombinant Adenovirus E1Adl01/07

The in-frame deletion mutations dl1101 and dl1107, as described in Jelsma *et al.*, (1998) Virology 163, 494-502, were constructed using the oligonucleotide site directed technique of Zoeller and Smith (1984) *DNA* 3, 479-488, as modifed by Kunkel (1985) *PNAS* 82, 488-492. All of the reagents, bacterial strains, and M13 vectors used for mutagenesis were provided in the Muta-Gene *in vitro* mutagenesis kit

(commercially availble from Bio Rad, Hercules, CA). The M13 template DNA, used for mutagenesis of the E1A region, contained Ad5 sequences from nucleotide positions 22-1339 inserted between the BamH1 and Xba1 restriction enzyme sites in the multiple cloning sequence of M13mp19. The bacteriophage construct produced, named M13mp19E1A, was then propagated in *dut ung E. coli* bacterial strain CJ236 which results in an occasional incorporation of uracil in place of thymidine in the newly synthesized DNA. The oligonucleotides, for construction of the E1 mutants, were synthesized to consist of sequences of either 11 or 12 nucleotides of Ad5 sense DNA on either side of the sequence that was to be removed.

For the mutagenesis reaction, the mutagencic oligonucleotides were first phosphorylated at the 5' end, and then annealed to uracil containing M13mp11E1A single-stranded template DNA. The annealed primer/template reactions were incubated with T4 DNA polymerase, T4 DNA ligase and deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) to synthesize a complementary strand containing the E1A mutation of interest. The complementary strand synthesis reaction were then transfomed into the *ung*$^+$ wild type host bacterial strain, MV1190. After transformation the parental M13mp19E1A DNA strand, which contains uracil, cannot be replicated efficiently in MV1190. Therefore the replicative form double strand DNA containing the E1A mutation of interest is enriched. M13mp19E1A phage DNA from potential E1A mutants was first screened by restriction enzyme analysis and then by DNA-sequencing, in both strands, to confirm the desired E1A-mutations.

Construction of the E1Adl01/07adenovirus was carried out by using homologous recombination in the adenovirus E1-region containing 293 cell line by the method of McGory, *et al.*, (1988) Virology 163, 614-617. This method requires two plasmids, one a viral plasmid containing the entire wtAd5 genome, and the other a transfer plasmid containing an E1A gene with the dl01/07 double E1A-mutant . The viral plasmid used for this work was pJM17, a non-infectious 40 kb plasmid, containing the entire Ad5dl309 genome in which the 4.4kb plasmid, pBX, is inserted in the unique Xba1 site. The transfer plasmid used, pLE2 contains wtAd5 sequences from 22-1774 cloned in the tetracycline gene of pBR322 Jelsma *et al.*, (1988)*Virology* 163, 494-502. For transfer of the E1A dl1101 and dl1107 E1a-mutants from the

M13mp19 background in which they were constructed, wild E1A restriction enzyme fragments in pLE2 were replaced with cognate mutated E1A fragments from M13mp19E1dl1101 and M13mp19E1Adl1107 to create pLE2E1Adl01/07. For recombination to produce adenovirus E1Adl01/07 the viral plasmid, pJM17 and pLE2E1Adl01/07 were cotransfected into 293 cells by calcium phosphate mediated transfection. After 5 hours the precipitate was rinsed and the cells were overlayed with growth medium containing agarose to isolate viral plaques. At 7-10 days after the initial transfection viral plaques were isolated, plaque purified two times, and subsequently viral DNA was screened using restriction enzyme analysis and DNA sequencing. Viral stocks were purified by double cesium chloride gradients and quantitated by column chromatography as described in Huyghe, *et al.* (1995) Human Gene Therapy 6:1403-1416.

Example 2. Construction of E1Bdl55K

The E1Bdl55K adenovirus was prepared by using oligonucleotide site directed mutatgenesis in substantial accordance with the teaching of Example 1 above. This procedure was used to introduce restriction enzyme nuclease cleavage sites in the E1B 55K coding region. The first site was introduced by modifying positions 2247 and 2248 of the wild type Ad5 genome wherein a guanine^{2247} was replaced with a thymidine and thymidine^{2248} replaced with cytosine (respectively) to introduce a EcoR1 cleavage site. This results in a modification of the E1B coding sequence at position 77 from valine to serine. A second resriction site was introduced at position 3272 wherein thymidine^{3272} was replaced with cytosine site (silent mutation) to introduce an XhoI site. The new restriction enzyme sites were used in a restriciton enzyme digest with EcoRI and Xho1. The EcoRI and XhoI sites were rejoined with a small polylinker cassette to introduce the polylinker isolated from the pBlueScriptSK (Stratagene, San Diego, CA). The resulting E1B mutation results in a coding sequence encoding the first 76 amino acids of the E1B55K protein followed by 18 missense amino acids resulting in a non-functional deleted E1B protein.

Example 3. In Vivo Mouse Model

On day 0, 30 athymic nude-nu mice (Harlan-Sprague-Dawley, Indianapolis IN) were injected in each flank with approximately 1x10^7 C33A cervical carcinoma cells in

200 microliters of Dulbecco's Modified Eagle Medium (DMEM). C33A cells are derived from human cervical carcinoma tissue and possess a p53 negative, Rb negative genotype and were obtained from the American Type Culture Collection. The tumors were allowed to grow for 6 days at which time they had reached a palpable size of approximately 100 mm^3. The animals were randomized by tumor size into 6 groups of 5 animals each. Each animal was intratumorally injected with $2.5x10^9$ particles of each different adenovirus construct in 60 microliters of PBS on Day 7, 8, 9, 10, and 11 following C33A administration. Each injection was divided among the four tumor quadrants. Tumors size was determined on Day 6, 9, 13, 16, 20, 23, 27 30 and 34 following C33A injection.

Example 4. Construction of E1Adl01/08

The recombinant adenovirus E1Adl01/08 is prepared in substantial accordance with the teaching of Example 1 above except that the transfer plasmid incorporates an in-frame deletion mutant to eliminate amino acids 124-127 of the E1A 289R and 243R proteins.

Example 5. Construction of E1Ad43/08

The recombinant adenovirus E1Adl43/08 is prepared in substantial accordance with the teaching of Example 1 above except that the transfer plasmid incorporates an in-frame deletion mutant to eliminate amino acids 38-60 and amino acids 124-127 of the E1A 289R and 243R proteins.

Example 6. Construction of E2F-E1Adl01/07

The recombinant adenovirus E2F-E1Adl01/07 describes a virus where an E2F pathway responsive promoter is used to drive expression of the modified E1a coding sequence. The base vector (E1Adl01/07) is prepared in accordance with the teaching of Example 1 above. However, the transfer plasmid is modified to replace the E1a promoter function with the E2F responsive promoter described in Parr, *et al.* (1997, Nature Medicine 3:1145-1149).

Example 7. Construction of E2F-E1Adl01/07 p53CON-E2F-RB

The recombinant adenovirus E2F-E1Adl01/07 p53CON-E2F-RB is the E2F-E1Adl01/07 vector prepared in substantial accordance with the teaching of Example 9 above and further comprises an expression cassette encoding a p53 pathway responsive

promoter driving expression of an inhibitor of viral replication (E2F-Rb). The expression cassette encoding a p53 pathway responsive promoter driving expression of an inhibitor of viral replication (E2F-Rb) is prepared as follows. A synthetic DNA sequence encoding a fusion protein comprising amino acids 95-286 of wild type E2F and amino acids 379-928 of the wild type Rb protein as described in European Patent Application No. 94108445.1 published December 6, 1995 (Publication number. 0 685 493 A1), with appropriate restriction sites is prepared by conventional techniques. The p53 pathway responsive promoter 53-CONis a synthetic p53 pathway responsive promoter constructed by insertion of two synthetic p53 consensus DNA binding sequences (as described in Funk, *et al.* (1992) Mol.Cell Biol. 12:2866-2871) upstream of the SV40 TATA box. p53CON can be constructed by annealing compliementary oligonucloeitides and p53 responsive promoters can be constructed by cloning in plasmid pGL3-promoter luciferase vector (commercially available from ProMega). This sequence is introduced into the E3 region of the dl309 adenvirus by homomogous recombination.

Example 8. E2F-E1Adl01/07 -SRE-E2F-Rb

Vector E2F-E1Adl01/07-SRE-E2F-Rb is prepared in substantial accordance with the teaching of Example 10 above. However, in place of the p53 pathway responsive promoter p53CON, a TGF-beta pathway respoonsive promoter (SRE) is used to drive expression of the E2F-Rb fusion protein. SRE refers to a synthetic TGF–β response element comprising a repeat of 4 of the Smad-4 DNA binding sequences (GTCTAGAC as described in Zawel, *et al.* (1988) Mol. Cell 1:611-617. The SRE response element may be generated by annealing complimentary oligonucleotides encoding the Smad-4 binding sequences and cloning in plasmid pGL#3 - promoter luciferase vector (commercially availabe from ProMega).

CLAIMS

I claim:

1. A method of ablating neoplastic cells in a population of normal cells contaminated by said neoplastic cells by the administration to said population of cells a selectively replicating recombinant adenovirus which contains modifications to the E1a coding sequence so as to produce E1a gene products which are deficient in binding to one or more p300 protein family members and one or more Rb protein family member protein but express a modified 289R protein retaining the transactivating function of the E1a CR3 domain.

2. The method of claim 1 wherein said vector contains a deletion corresponding to amino acids 4-25 of the E1a 243R and 289R proteins.

3. The method of claim 1 wherein said vector contains a deletion corresponding to amino acids 38-60 of the E1a 243R and 289R proteins.

4. The method of claim 2 wherein said vector further comprises a deletion corresponding to amino acids 111-123 of the E1a 243R and 289R proteins.

5. The method of claim 2 wherein said vector further comprises a deletion corresponding to amino acids 124-127 of the E1a 243R and 289R proteins.

6. The method of claim 3 wherein said vector further comprises a deletion corresponding to amino acids 111-123 of the E1a 243R and 289R proteins.

7. The method of claim 3 wherein said vector further comprises a deletion corresponding to amino acids 124-127 of the E1a 243R and 289R proteins.

8. The method of claim 1 wherein said method comprises the administration of a pharmaceutically acceptable formulation of said vector to a mammal.

9. The method of claim 8 wherein said mammal is a human.

10. The method of claim 9 wherein said formulation further comprises a delivery enhancing agent.

11. The method of claim 1 wherein said vector is administered to a stem cell product *ex vivo*.

12. A pharmaceutical formulation comprising a selectively replicating recombinant adenovirus which contains modifications to the E1a coding sequence so as to produce E1a gene products which are deficient in binding to one or more p300

protein family members and one or more Rb protein family member protein but express a modified 289R protein retaining the transactivating function of the E1a CR3 domain.

13. The formulation of claim 12 wherein said vector further comprises a deletion corresponding to amino acids 4-25 of the E1a 243R and 289R proteins and amino acids 111-123 of the E1a 243R and 289R proteins.

14. The formulation of claim 12 wherein said vector further comprises a deletion corresponding to amino acids 4-25 of the E1a 243R and 289R proteins and amino acids 124-127 of the E1a 243R and 289R proteins.

15. The formulation of claim 12 wherein said vector further comprises a deletion corresponding to amino acids 38-60 of the E1a 243R and 289R proteins and amino acids 111-123 of the E1a 243R and 289R proteins.

16. The formulation of claim 12 wherein said vector further comprises a deletion corresponding to amino acids 38-60 of the E1a 243R and 289R proteins amino acids 124-127 of the E1a 243R and 289R proteins.

17 The formulation of claim 12 further comprising a delivery enhancer.

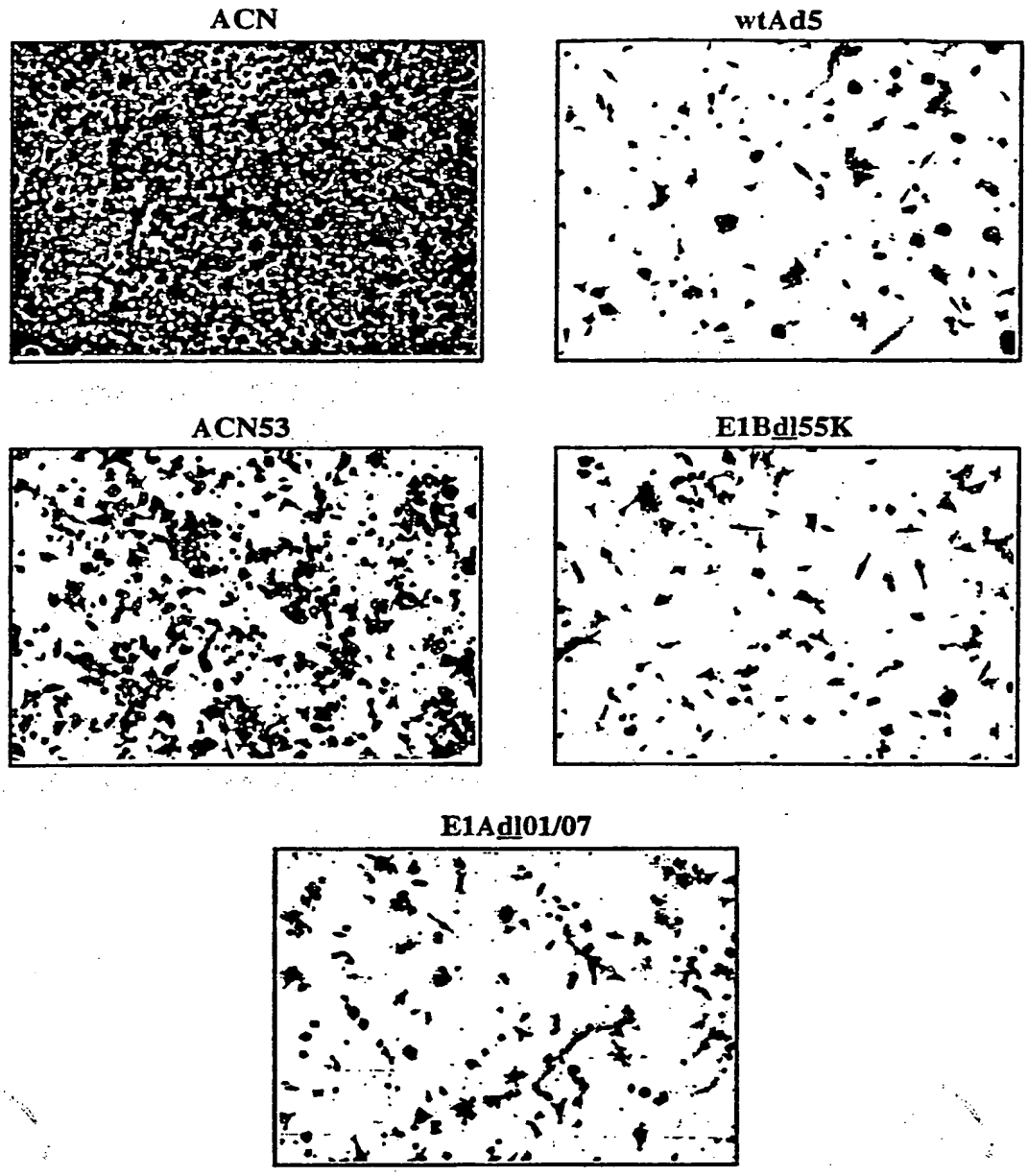


FIGURE 1

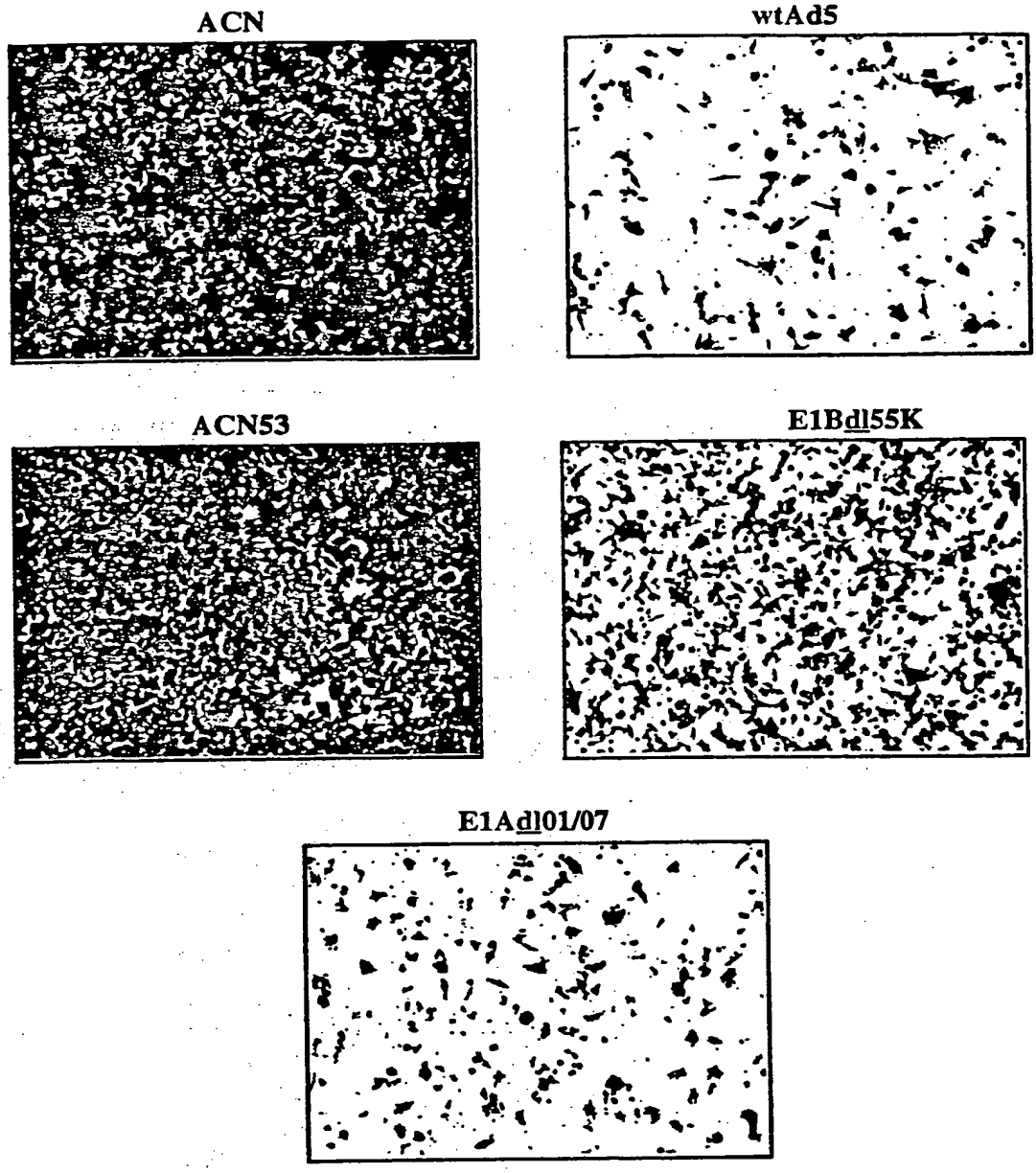


FIGURE 2

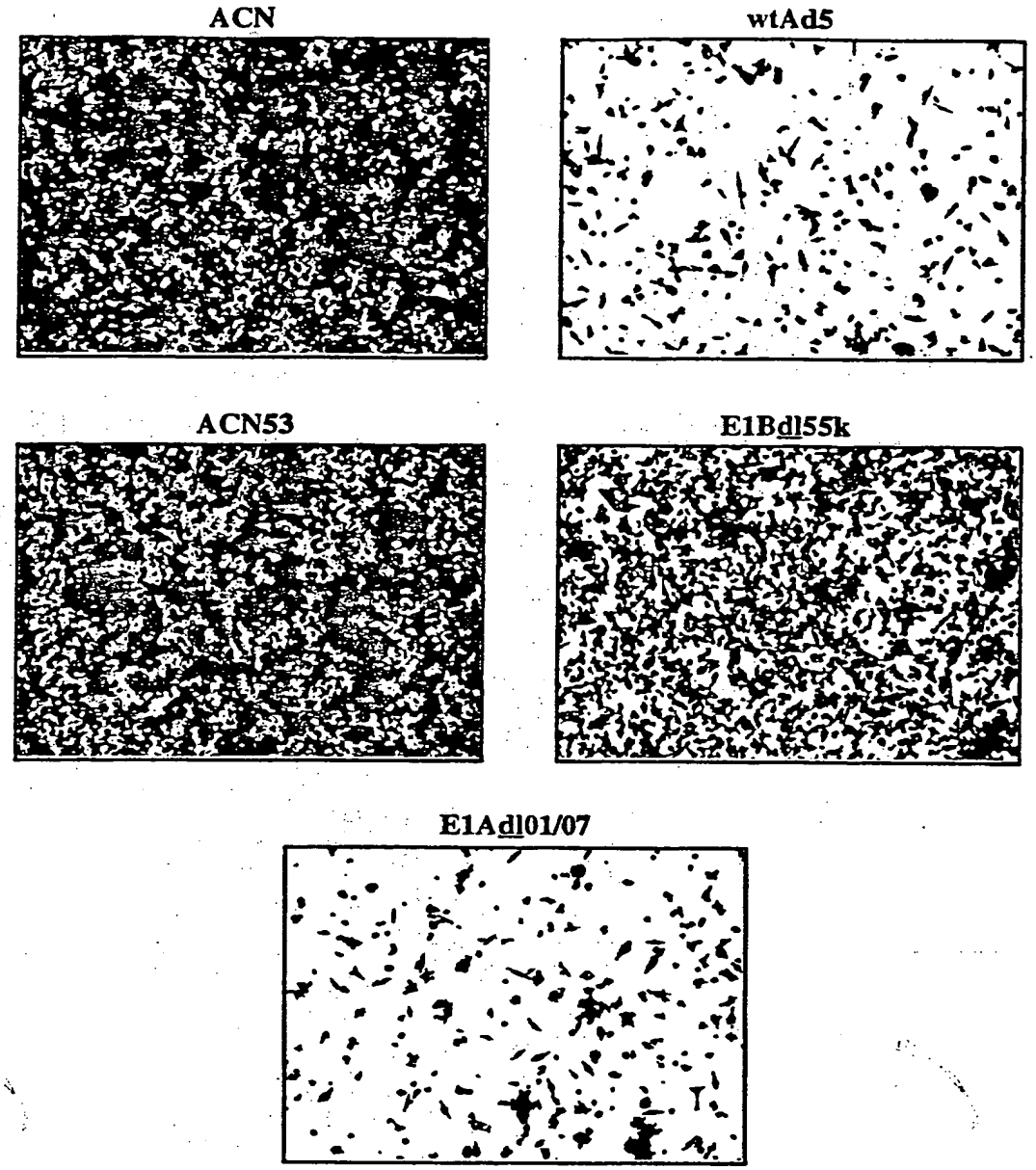


FIGURE 3

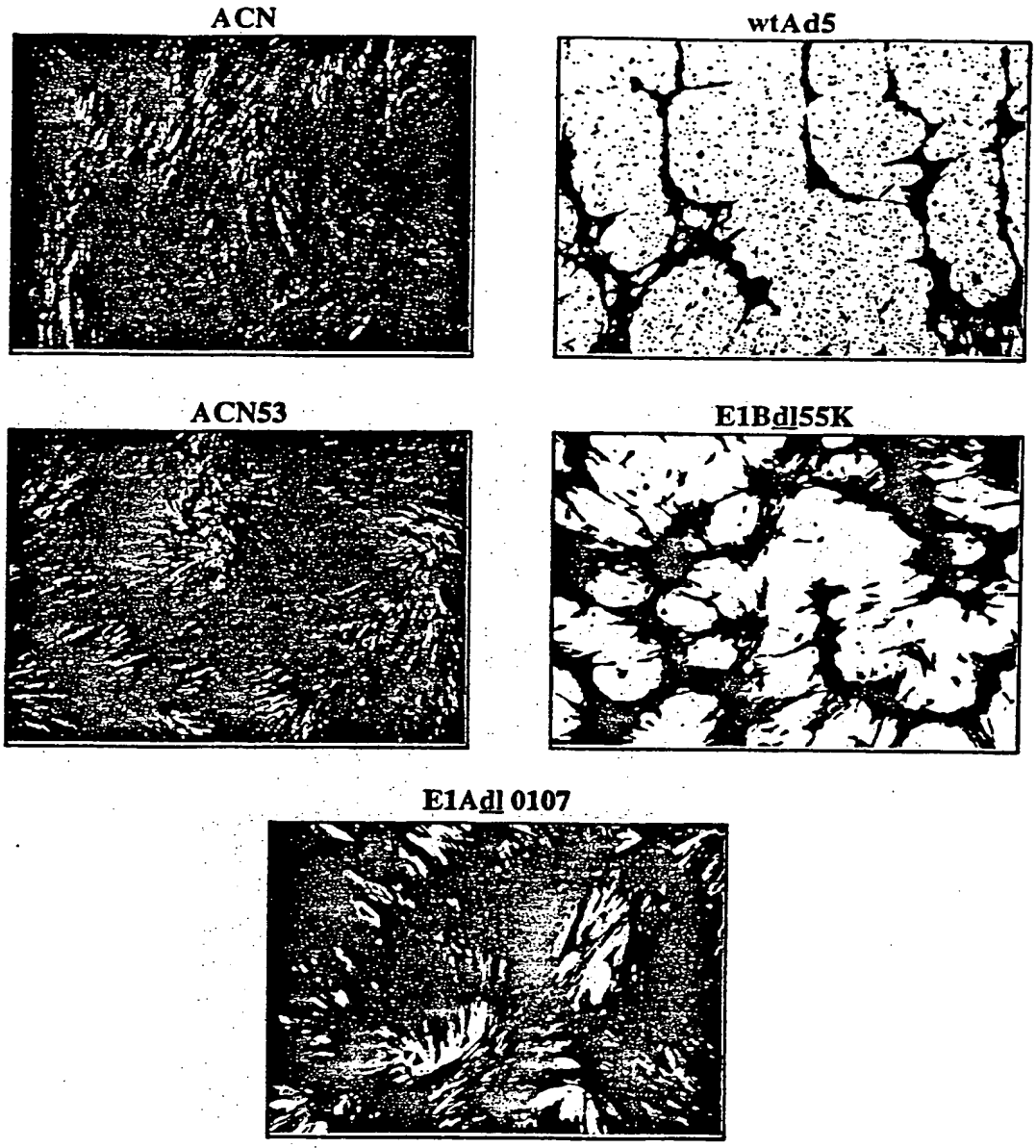


FIGURE 4

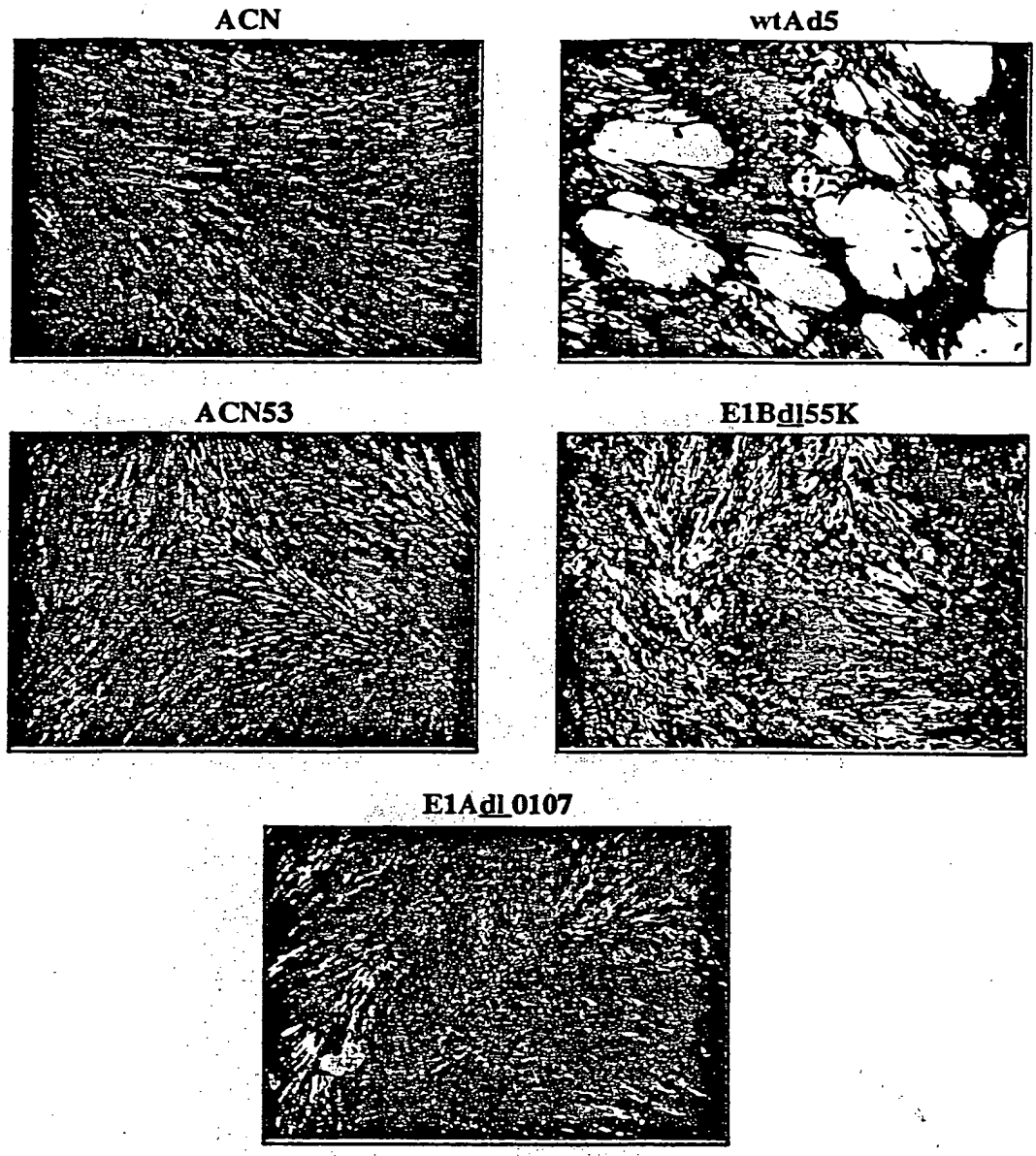


FIGURE 5

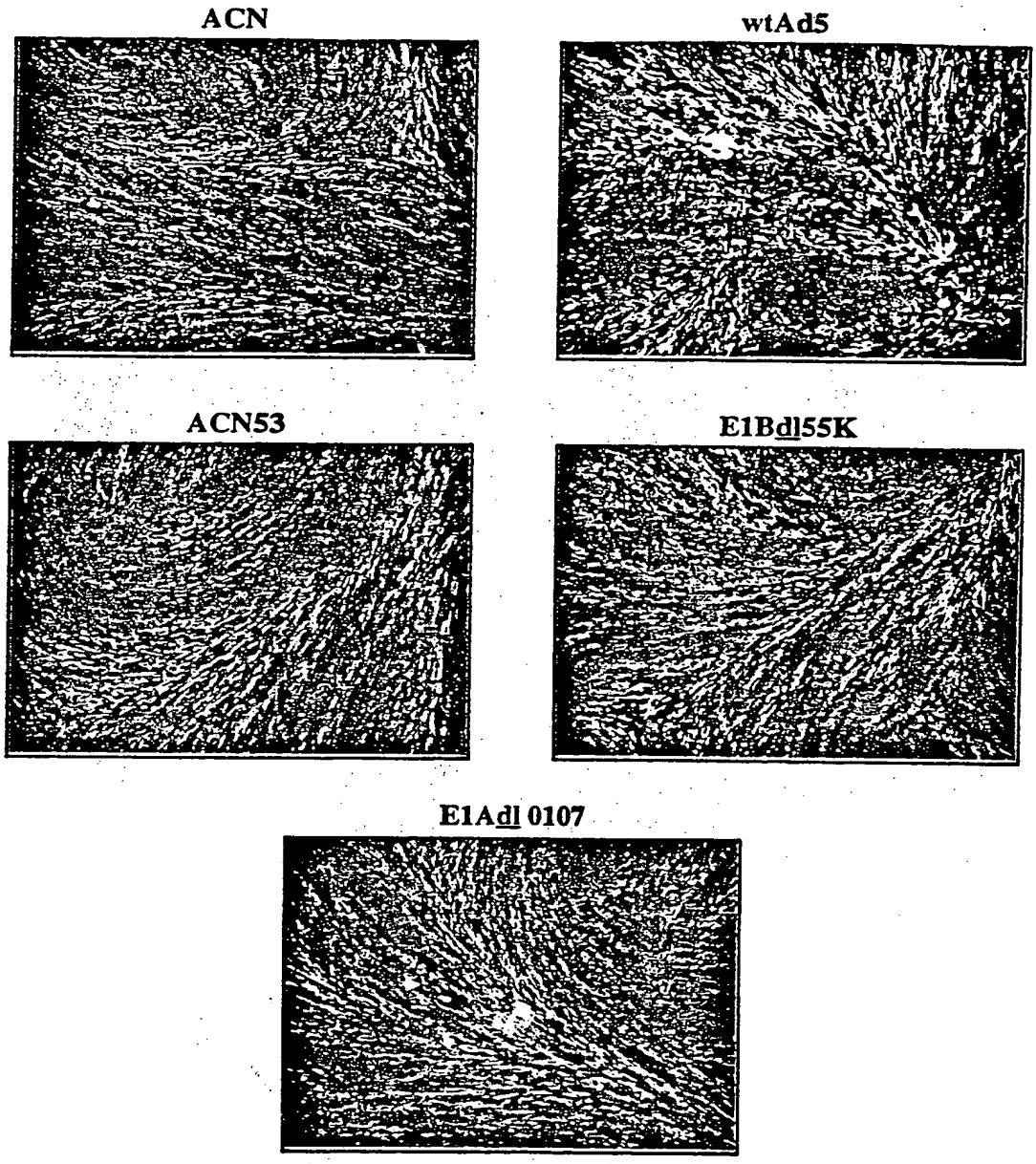


FIGURE 6

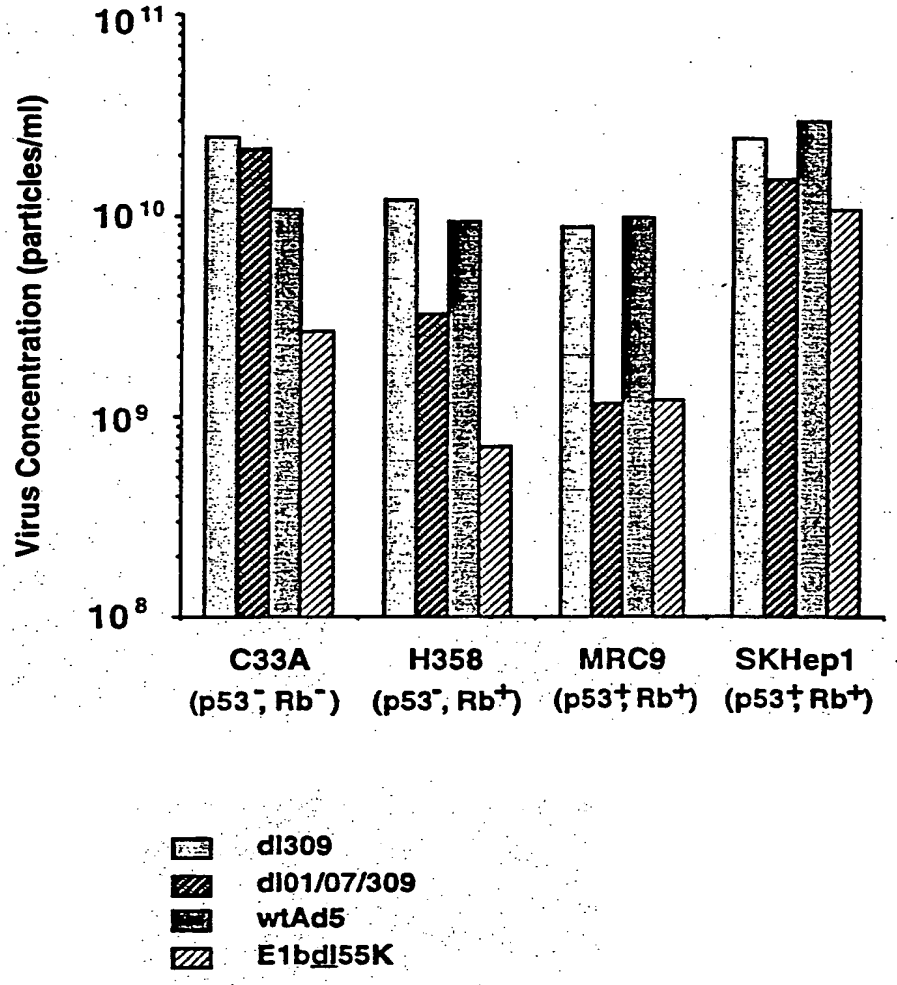

Virus Concentration (particles/ml)
10 11
10 10
10 9
10 8
C33A
(p53-; Rb-)
H358
(p53-; Rb+)
MRC9
(p53+; Rb+)
SKHep1
(p53+; Rb+)
dl309
dl01/07/309
wtAd5
E1bdl55K

FIGURE 7

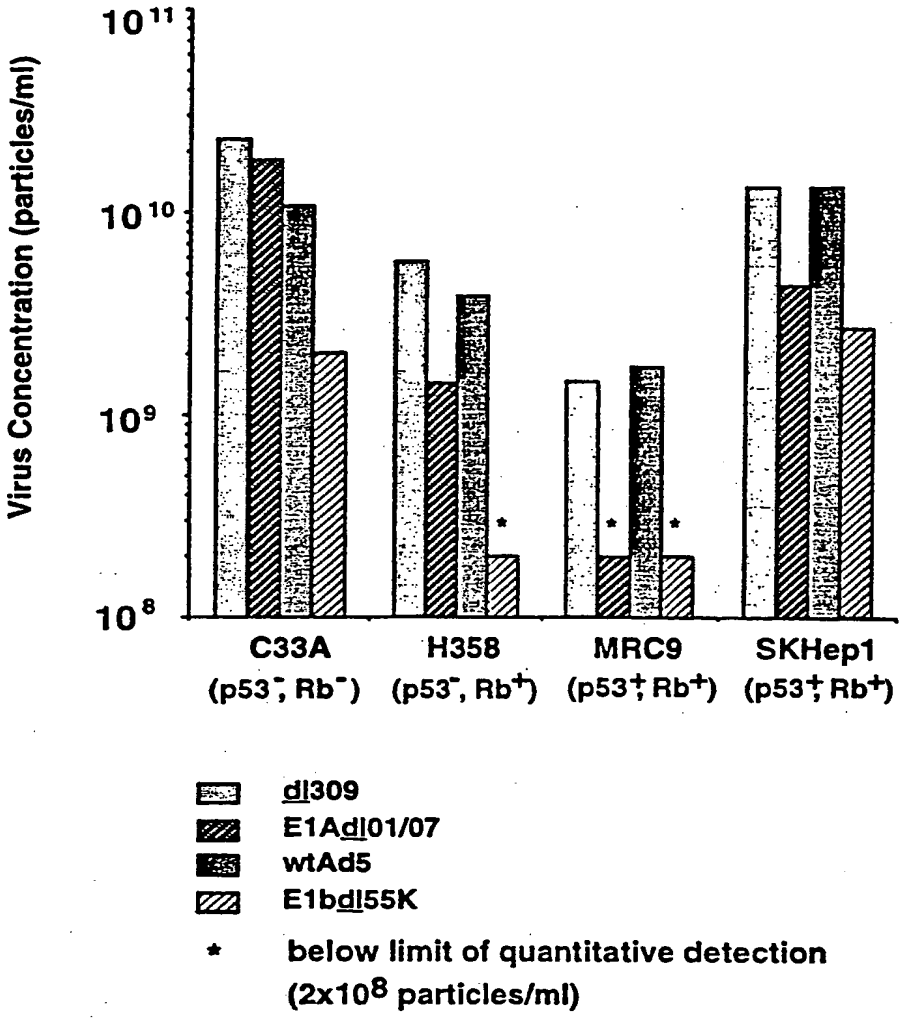

Virus Concentration (particles/ml)
10^11
10^10
10^9
10^8
C33A (p53-, Rb-)
H358 (p53-, Rb+)
MRC9 (p53+, Rb+)
SKHep1 (p53+, Rb+)
dl309
E1Adl01/07
wtAd5
E1bdl55K
* below limit of quantitative detection (2x10^8 particles/ml)

FIGURE 8

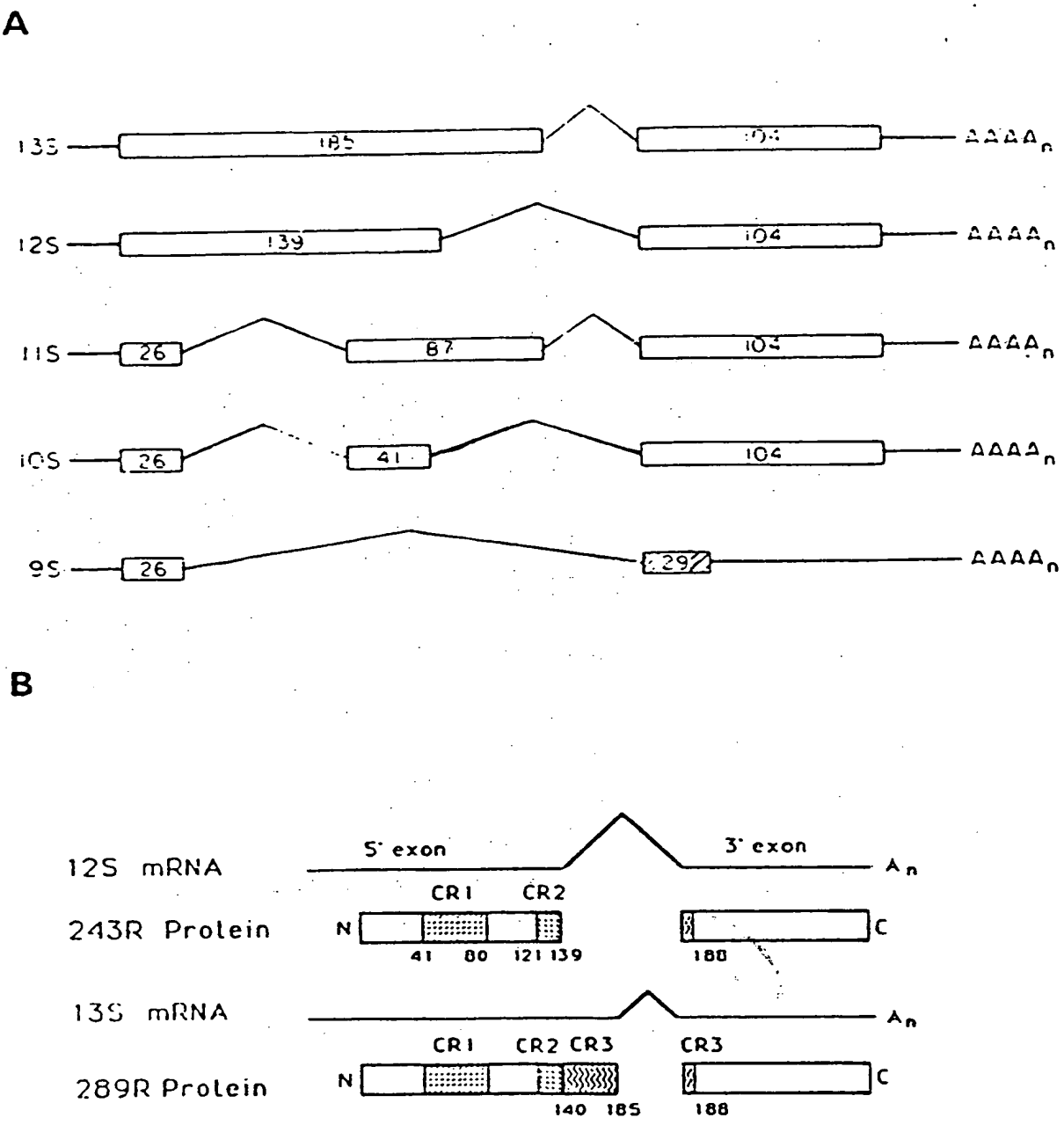


Figure 9

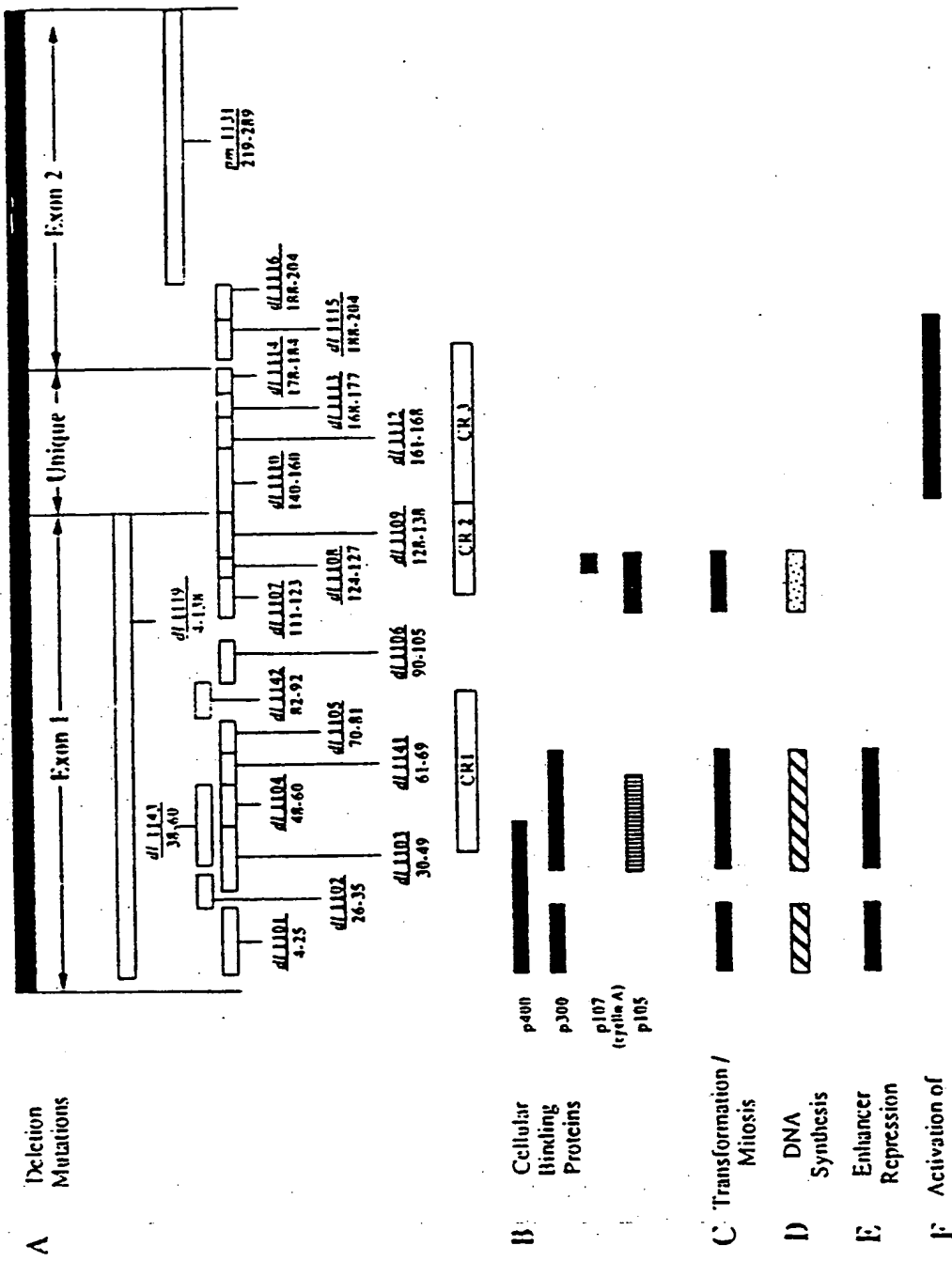


Figure 10

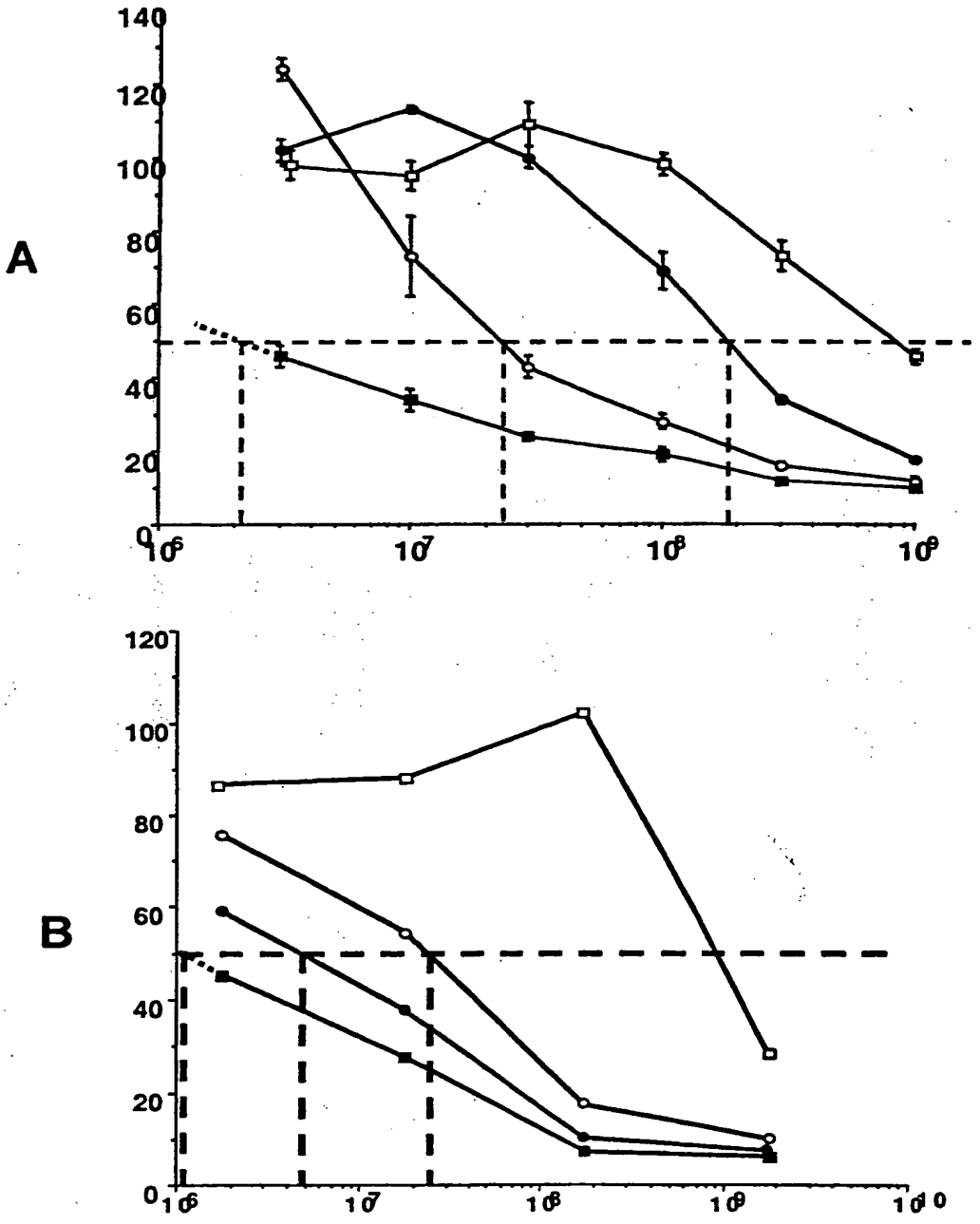
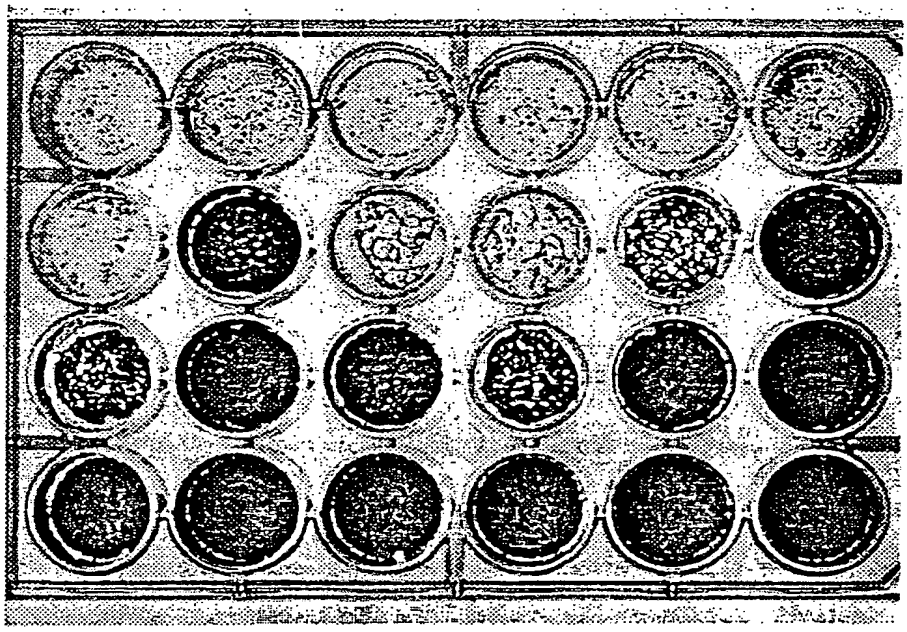


Figure 11

A



B

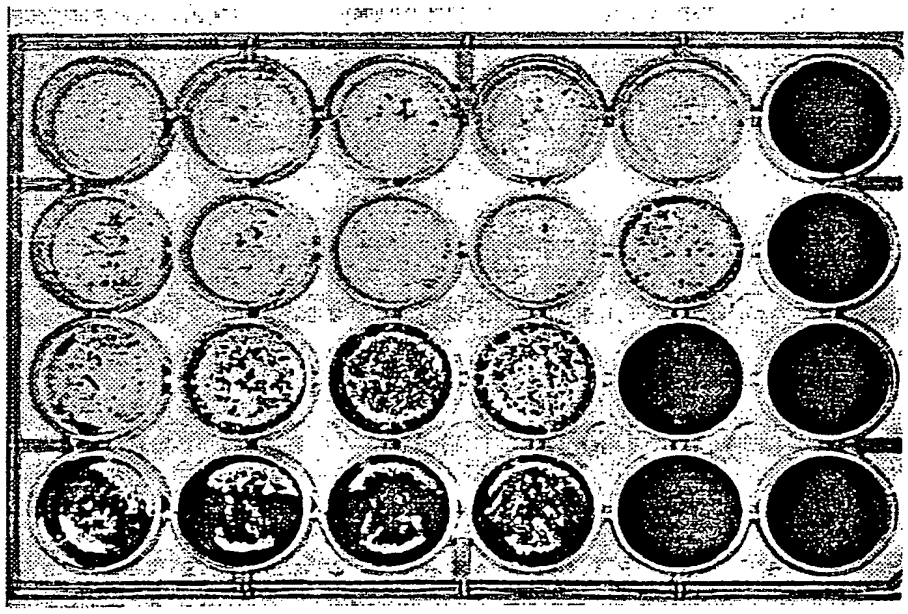


Figure 12

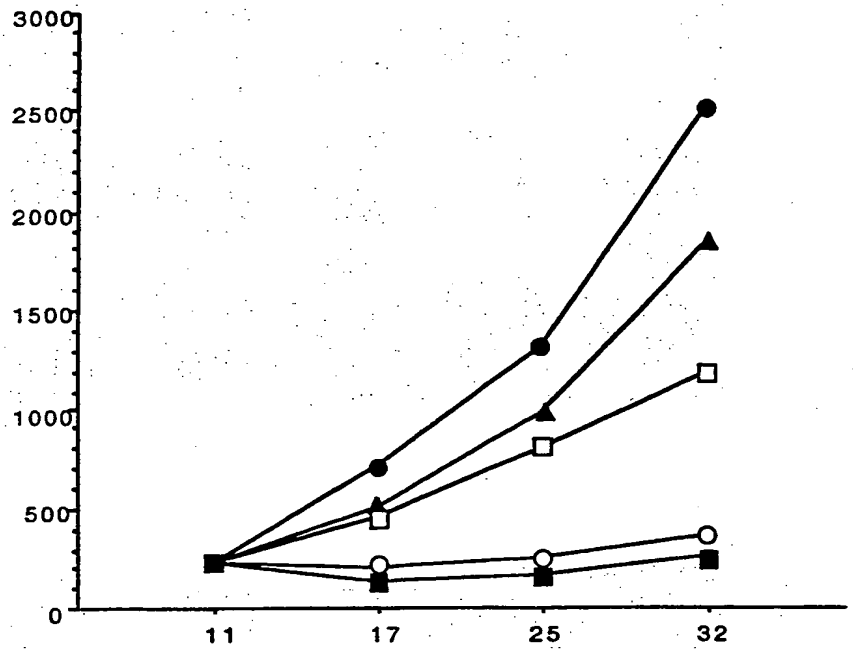


Figure 13

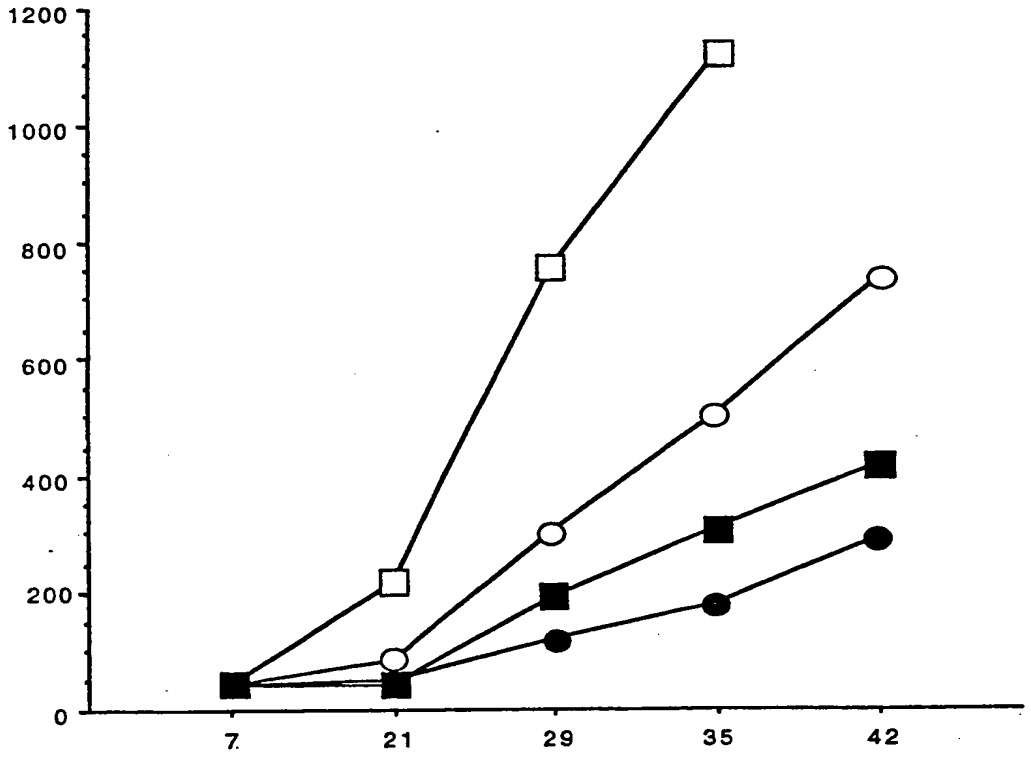


Figure 14

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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20 April 2000 (20.04.2000)

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(21) International Application Number: PCT/US99/21451

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(25) Filing Language: English

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09/172,684 15 October 1998 (15.10.1998) US

(71) Applicant: CANJI, INC. [US/US]; 3525 John Hopkins Court, San Diego, CA 92121 (US).

(72) Inventor: HOWE, John, A.; 12466 Cavallo Street, San Diego, CA 92130 (US).

(74) Agents: MURPHY, Richard, B. et al.; Schering-Plough Corporation, Patent Dept., K-6-1 1990, 2000 Galloping Hill Road, Kenilworth, NJ 07033-0530 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL, IN, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LU, LV, MA, MD, MG, MK, MN, MX, NO, NZ, PL, PT, RO, RU, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UZ, VN, YU, ZA.

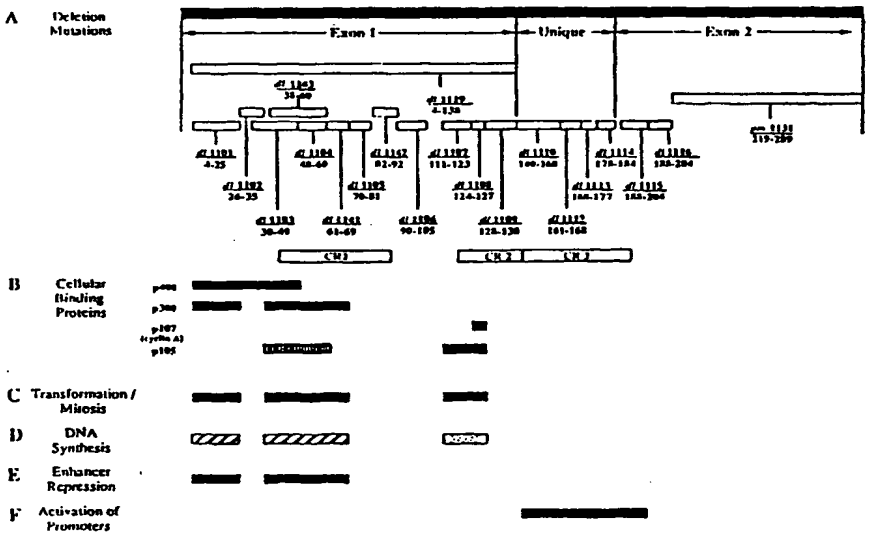
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
30 November 2000

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: RECOMBINANT E1A DELETED ADENOVIRAL VECTORS



(57) Abstract: The present invention is directed to recombinant adenoviral vectors capable of replication under particular host cell conditions. In particular, the present invention provides adenoviruses containing modifications to the E1a region which have therapeutic and diagnostic applications. The vectors of the present invention are capable of replication and lysis of neoplastic cells. The vectors may optionally include modifications to the genome so as to impart specific replicative or targeting functions. The present invention also provides pharmaceutical formulations of such vectors. The present invention further provides methods of use of such vectors. The present invention further provides methods of preparing the vectors.

WO 00/22136 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/21451

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/34 C12N15/861 C07K14/075 A61K48/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 94 18992 A (ONYX PHARMACEUTICALS) 1 September 1994 (1994-09-01) page 4, line 23-38 page 6, line 4-25 page 17, line 24-37; figure 1 page 18, line 1-9 page 18, line 35 -page 19, line 11 page 28, line 12 -page 31, line 11 example 1 claims 1-3,5,7-10,13,14,16 --- -/--	1,8-12, 17 2-5,7, 13,14,16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 August 2000	13. 09. 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

(July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/21451

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	HOWE J A ET AL.: "Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis" PROC. NATL. ACAD. SCI. USA, vol. 87, August 1990 (1990-08), pages 5883-5887, XP002134798 abstract figure 1; table 1 page 5886, right-hand column, line 27-40 ---	2-5,7, 13,14,16
Y	BARBEAU D ET AL.: "Functional interactions within adenovirus E1A protein complexes" ONCOGENE, vol. 9, no. 2, February 1994 (1994-02), pages 359-373, XP000891798 abstract figure 5; table 1 ---	2,4,5, 13,14
A	JELSMA T N ET AL.: "Use of deletion and point mutants spanning the coding region of the adenovirus 5 E1A gene to define a domain that is essential for transcriptional activation" VIROLOGY, vol. 163, no. 2, April 1988 (1988-04), pages 494-502, XP000891615 cited in the application abstract figure 2; table 1 ---	1,2,4,13
A	BAYLEY S T ET AL.: "Adenovirus E1A proteins and transformation (Review)" INTERNATIONAL JOURNAL OF ONCOLOGY, vol. 5, no. 3, September 1994 (1994-09), pages 425-444, XP000877368 cited in the application abstract figure 1 -----	1

INTERNATIONAL SEARCH REPORT

Ir. .national application No.
PCT/US 99/21451

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-11 (as far as they concern or are related to in vivo methods) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

As a result of the prior review under R. 40.2(e) PCT, no additional fees are to be refunded.

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[X] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,8-12,17 (all partially); 2,4,5,13, 14 (all completely)

A method of ablating neoplastic cells in a population of normal cells contaminated by neoplastic cells, by the administration to said population of cells a selectively replicating recombinant adenovirus which contains modifications to the E1a coding sequence so as to produce E1a gene products which are deficient in binding to one or more p300 protein family members and to one or more retinoblastoma (pRb) protein family members, but expresses a modified 289R protein retaining the transactivating function of the E1a CR3 domain, said adenoviral vector containing a deletion corresponding to the amino acids 4-25 of the E1a 243R and 289R proteins, preferably comprising a further deletion corresponding to amino acids 111-123 or to amino acids 124-127 of said proteins. Also claimed are further administration methods, and pharmaceutical formulations comprising said adenoviral vectors.

2. Claims: 1,8-12,17 (all partially); 3,6,7,15, 16 (all completely)

A method of ablating neoplastic cells in a population of normal cells contaminated by neoplastic cells, by the administration to said population of cells a selectively replicating recombinant adenovirus which contains modifications to the E1a coding sequence so as to produce E1a gene products which are deficient in binding to one or more p300 protein family members and to one or more retinoblastoma (pRb) protein family members, but expresses a modified 289R protein retaining the transactivating function of the E1a CR3 domain, said adenoviral vector containing a deletion corresponding to the amino acids 38-60 of the E1a 243R and 289R proteins, preferably comprising a further deletion corresponding to amino acids 111-123 or to amino acids 124-127 of said proteins. Also claimed are further administration methods, and pharmaceutical formulations comprising said adenoviral vectors.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/21451

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9418992 A	01-09-1994	AT 178490 T	15-04-1999
		AU 682854 B	23-10-1997
		AU 6272294 A	14-09-1994
		CA 2152941 A	01-09-1994
		DE 69417734 D	12-05-1999
		DE 69417734 T	07-10-1999
		EP 0689447 A	03-01-1996
		EP 0931830 A	28-07-1999
		ES 2134346 T	01-10-1999
		GR 3030385 T	30-09-1999
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		US 5972706 A	26-10-1999
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		US 5677178 A	14-10-1997
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